

IDENTIFICATION, CHARACTERIZATION, AND REGULATION OF PORCINE
OVIDUCTAL PLASMINOGEN ACTIVATOR INHIBITOR-1 AND FUNCTIONAL
ANALYSIS OF OVIDUCTAL-SPECIFIC SECRETORY GLYCOPROTEIN

By

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This Dissertation is dedicated to Brenda and Jerry Kouba.

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In mammals the oviduct *de novo* synthesizes and secretes numerous proteins that may act to facilitate the processes of fertilization and early cleavage-stage embryonic development. Several of these proteins have been shown to be steroid-modulated and have cycle-specific expression. However, the majority of these proteins remain unidentified and have only been characterized in a limited fashion by molecular weight and isoelectric point. Functional data relating to these proteins is especially lacking. The first objective was to identify and characterize the major *de novo* secretory product of the isthmus. The second objective was to examine potential functions of the well-characterized pOSP during *in vitro* fertilization and embryo culture.

The major *de novo* secretory product of the isthmus was identified as plasminogen activator inhibitor-1 (PAI-1). It was demonstrated that this protein is localized to oviductal epithelium and packaged into putative secretory granules for exocytosis. Upon

release into the oviductal lumen, PAI-1 was shown to associate with oviductal oocytes, embryos, and spermatozoa. Characterization of this protein revealed that (1) estrogen inhibits mRNA and protein expression, (2) mRNA and protein is greatest during fertilization and early embryo development, and (3) once activated, PAI-1 can form a complex with uPA and inhibit its activity. In addition, PAI-1 levels are greatest when PA activity was highest. These results suggest an important role for the regulation of the plasminogen/plasmin system during fertilization and early embryonic development.

Functional analysis of pOSP revealed several potential actions of this protein during fertilization. It was demonstrated that pOSP significantly reduced the incidence of polyspermy in pig oocytes fertilized *in vitro*. After fertilization in the presence of pOSP, a reduction in the number of sperm cells attached to the zona pellucida was observed. This may be a potential mechanism whereby pOSP reduces the number of sperm cells that can attach and penetrate an egg. Pig oocytes fertilized in the presence of pOSP had increased blastocyst development compared to the control. Therefore, the reduction in the rate of polyspermic fertilization may have resulted in enhanced development to blastocyst. The pOSP reduction in polyspermy could be inhibited by anti-pOSP indicating that the decreased incidence of polyspermic fertilization was specific to pOSP.

The characterization and functional analysis of these two proteins, PAI-1 and pOSP, indicates an important relationship between their expression, association with the oocyte or embryo, and potential actions during fertilization and development.

CHAPTER 1 INTRODUCTION

This dissertation will attempt to enlighten and convey to the reader the importance of the mammalian oviduct as not only a conduit for the transport of gametes but also as an important reproductive organ that contributes to the well-being and development of the embryo. Numerous studies have been done indicating that a pregnancy can be established without the presence of features and structures that are located within or are specific to the oviduct. Perhaps the most pervasive example is that of *in vitro* fertilization (IVF), in which an oocyte is matured, fertilized, and developed *in vitro* with subsequent transplantation of the embryo directly into the uterus, resulting in a successful pregnancy. Techniques such as IVF, which remove the oocyte and spermatozoa from contact with the tubule, have generated much debate on the question of whether the mammalian oviduct is an essential part of the female reproductive tract.

Over the past decade, numerous advances have been accomplished for embryonic development *in vitro*, especially during oocyte maturation and fertilization. These advances have been primarily due to careful formulation of culture medium and use of co-culture with oviductal epithelial cells. Interpretation of these studies has led to two prevalent hypotheses. One view is that the oviduct has a more passive role in terms of establishing an environment with an optimum pH, temperature, osmotic pressure, nutrients, and oxygen tension for fertilization and early cleavage-stage development. The second is the oviduct has a more active role, investing energy into the expression and

synthesis of molecules, which may facilitate or regulate these events. Evidence to date suggests that these two views are not mutually exclusive but are cooperative in the formation of a microenvironment, which sustains fertilization and early cleavage-stage development. Perhaps the importance of this reproductive organ has to be viewed with respect to its importance in the establishment of a species population. An insightful paragraph by Dr. Ronald H. F. Hunter in the book The Fallopian Tubes (1988) touches on this theory:

For most biologists, successful reproduction needs to be considered in population terms, and all features that contribute to the stability of a breeding population, and thereby to perpetuation of the species, need to be brought to mind. It is in this light that the many specific contributions of the Fallopian tubes are best interpreted. Whilst individual morphological and biochemical components may not be of overriding importance in themselves for the establishment of single pregnancies, together they can be viewed as contributing to the successful maintenance of a breeding population.

Although embryonic development *in vitro* has recently seen significant advances, scientists are still far from reaching a degree of development and viability similar to that seen *in vivo*. In virtually every reported case, cultured embryos exhibit reduced cell numbers, decreased viability with increasing duration of culture, and reduced development to later stages when compared with their counterparts growing *in vivo* [Bavister, 1988]. These observations indicate that some important characteristics of the embryonic *milieu* are missing from the artificial culture environment. Peptide and macromolecules are the oviductal constituents most suspected of having specific embryotrophic actions, particularly *de novo* synthesized and secreted products of the epithelium. The first objective was to identify an unknown major *de novo* secretory

product of the porcine isthmus. Sequence analysis revealed this protein to be porcine plasminogen activator inhibitor-1 (PAI-1). Experiments were designed to characterize this protein throughout the estrous cycle or early pregnancy and to evaluate its association with the pre-implantation embryo. The second part of this dissertation concerns the functional analyses of a well-characterized oviductal protein, porcine oviductal secretory glycoprotein (pOSP), during fertilization and early embryonic development. It may be that with the identification and functional analysis of specific oviductal molecules, conditions for *in vitro* development of embryos may come to mimic more closely that of the *in vivo* situation. This dissertation will present data that indicates a more active role for this reproductive organ in the successful establishment of pregnancy.

CHAPTER 2 LITERATURE REVIEW

Biology of the Oviduct

Historical Perspective

The first observations of the oviduct, date back to Aristotle (322-384 BC), who noted a convoluted structure connecting the uterus to the ovary. However, a common misinterpretation that perpetuated for several centuries was that the ducts transmitted female semen to the urinary bladder where it was excreted. Accurate anatomical descriptions were first made by Galen (AD 130-200), linking the ovaries to the uterus and describing their termination in the uterine horns of bicornuate domestic animals. However, his mistake was to perpetuate the concept that this tubule was a conduit for female semen, which was filtered by the ovary from the bloodstream. This notion was based upon his observation that the female Fallopian tubes were similar to the male seminal vessels.

The idea of a tubule which transmitted female semen persisted for over 14 centuries. Descriptive anatomical drawings were not available until the excellent works of Leonardo da Vinci (1452-1519) were published. Another anatomist, Andreas Vesalius, extensively characterized the anatomy of the Fallopian tube in 1543 in a seven-volume series known as *De Humani Corporis Fabrica*. His analyses of their function echoed that of the past by supporting the hypothesis that these structures were responsible

for the transport of female semen. The critical structure of the oviduct and relationship of this structure to the ovary and uterus were detailed by Fallopius, and due to his detailed description of the tubule his name was lent to the structure (Fallopian tube). The collected works of Harvey (1651), Van Horne (1668), and de Graaf (1672) established that female semen was a myth, but that instead an egg originates in the ovary (although the follicle was misinterpreted as the egg), and that this egg is transported into the uterus via the Fallopian tubes. It was not until significant advances in the technological development of the microscope were made near the end of the 17th century that a more careful scrutiny of the oviduct and its structure, came about. For an excellent review on the history of the oviduct see The Fallopian Tubes (1988), edited by Hunter and The Mammalian Oviduct (1969), edited by Hafez and Blandau.

Prenatal Development and Macroscopic Anatomy of the Oviduct

The oviducts of adult mammals are specialized structures that originate during organogenesis from the cranial region of the primitive Mullerian ducts [Price et al., 1969]. Johannes Muller first described the Mullerian ducts in 1830, yet far less attention has been directed toward the development of the oviduct in relation to the uterus. Although the development of the oviduct prenatally differs widely among mammalian species, several general statements can still be made. The oviduct undergoes morphogenetic and histogenetic changes that distinguish it from the uterus which include (1) coiling of the oviduct, (2) differences in size (diameter), (3) differentiation of epithelium and muscle, (4) formation of mucosal folds, (5) development of the fimbriae, and (6) development of the three segments – infundibulum, ampulla, and isthmus [Price et al., 1969]. For a detailed description of comparative development of the mammalian

oviduct, the reader is referred to The Mammalian Oviduct (1969), edited by Hafez and Blandau.

The paired oviducts connect within the uterus at the uterine tubule junction (UTJ) and link this structure to the ovary. The oviduct is convoluted in most species (except the rabbit and man), and is supported by the mesosalpinx, which is a part of the broad ligament [Nalbandov, 1969]. A specialized portion of the mammalian oviduct is often referred to as the fimbriae infundibulum, which forms a fringed open funnel. In the pig, which has open fimbriae, this is an intimate structure which encapsulates the ovary. In species such as mouse and rat this structure forms a sac around the ovary called the bursa ovarii. The fimbriae of the infundibulum are extremely motile and direct fluid movement into the oviduct during the time of ovulation. In the pig, unilateral removal of the infundibulum does not cause infertility as the contralateral fimbriae can redirect and capture oocytes from the peritoneal cavity [Nalbandov, 1969]. The segment adjacent to the infundibulum is referred to as the ampulla and has the largest diameter of the three segments of the oviduct. The intricate folding of the mucous membrane is also characteristic of this section. The diameter of the ampulla tapers down as it becomes the isthmus which, in the pig, connects into the uterus at the tip of the uterine horn. The pig oviduct averages 26-28 cm in length, with the isthmus comprising approximately 1/3 of the overall length with the majority being made up of the ampulla.

The portion of the isthmus which enters the uterus is referred to as the uterotubal junction (UTJ), and in most litter-bearing species such as the pig, it contains a high density of folds and finger-like processes (villi) [Hafez and Black, 1969]. These structures are arranged in such a fashion so as to prevent the passage of fluid and uterine

contents into the oviduct. Anderson (1927a) showed that in pigs, a greater amount of pressure is required to force uterine fluid into the oviducts when eggs are present (Day 2-4 of the estrous cycle) than during the luteal phase. This suggests that the UTJ maintains the oviductal environment protecting it from contamination by secretions from the uterus.

As reviewed by Hunter (1988), the pig oviduct contains an outer layer of longitudinal muscle and an inner layer of circular muscle. External to these muscle layers is a well-vascularized serosa layer of tissue. The isthmus portion of the oviduct has a greater amount of circular muscle, allowing for a greater contraction potential than the other two segments. The circular muscle layers of the isthmus and ampullary-isthmic junction are highly innervated with adrenergic nerve terminals originating in the ovarian or hypogastric plexus. The innervation of the ampulla and infundibulum is very poor compared to the isthmus, and is usually restricted to the walls of blood vessels. The high density of circular muscle and adrenergic innervation of the isthmus indicates an important role for this segment in the transport of gametes and embryos.

The lymphatic vessels of the pig oviduct follow the ovarian and uterine lymphatic drainage and show a similar concentration in the isthmus as was described for the sympathetic innervation of nerve bundles. Anderson's (1927b) study of the domestic pig, revealed that cyclical variations could be noted in lymphatic development and that these changes might be due to ovarian steroids. It has been suggested that the lymphatics might be involved in transmission of embryonic information to either the ovary or uterus, since the greatest amount of tubule resistance to the embryo is found within the isthmus.

The vascular supply to the oviduct is supported by the uterine and ovarian arteries, and varies according to the estrous cycle or pregnancy. The capillary bed and

larger blood vessels of the infundibulum and ampulla become engorged with blood close to the time of ovulation and are regulated by estrogen [Hunter, 1988]. This increase in blood supply may also account for the increased tubule secretion and transudation in the pig at estrus.

Microscopic Anatomy of the Oviduct

The architecture of the ampulla and isthmus is centered on an epithelium with numerous tubal mucosal folds that show cyclical changes in morphology. The actual space within the oviduct lumen is very negligible (unlike the lumen of a gland). Therefore, gametes often remain in intimate contact with the epithelial lining as opposed to floating free in an aqueous environment. This epithelial lining is composed of simple columnar and modified cells of the goblet cell type, which are secretory in nature. There are two distinct differentiated cell types within the oviduct, referred to as ciliated and non-ciliated (secretory), although this is not absolute as sometimes differentiating cells display both cilia and secretory granules [Jansen and Bajpai, 1982]. Interspersed throughout the ciliated cells are non-ciliated cells that contain electron-dense secretory granules that can be identified throughout the cell but are most often found in the apical region. These secretory granules exude their contents with greater frequency during ovulation, contributing to the decline in epithelial height shortly thereafter [Hunter, 1988]. In most domestic species, epithelial cell height and secretory activity are greatest at the time of ovulation.

Estrous Cycle of the Pig and Hormonal Regulation by Ovarian Steroids

The influence of ovarian steroid hormones on the oviductal environment has been well documented in several species for ciliogenesis, secretion, contractility, and

morphology of the tubal mucosa and musculature [Hunter, 1988]. It is difficult to discuss these processes in relation to the oviduct without first describing some aspects of the pig estrous cycle and its changing hormonal environment. A prepubertal gilt becomes sexually mature at 6-8 months of age and is described as polyestrous, which means a lack of seasonality in its reproductive cycle. The average cycle length for post-pubescent gilts is 21 days, with Day 0 being the onset of standing estrus. The average length of estrus is 2-3 days, with ovulation occurring 30-40 hours after the onset of estrus. A pig is a polytocous, or litter bearing, animal and ovulation occurs over a time period of 1 to 5 hours [Hunter, 1988]. Estrogen begins to rise several days prior to estrus (proestrus) and returns to basal levels by Day 2 of the estrous cycle [Karlhom et al., 1982]. Progesterone levels begin to rise by Day 2 of the estrous cycle in conjunction with development of the corpus luteum, reaching a peak around Days 14-15 and quickly declining thereafter [Karlhom et al., 1982].

Ovarian steroids are transported to the oviduct through the blood [Giorgi, 1980] which then bind to specific cytosolic receptors and are transported to the nucleus [Chan and O'Malley, 1976]. If steroid receptors are absent in the oviduct, the tissue becomes refractory to the action of that hormone. This provides a unique model in which oviductal function can be examined in intact and ovariectomized animals. Tissue concentrations of estradiol and progesterone receptors in the porcine oviduct were first examined by Stanchev et al. (1985). These researchers looked at both cytoplasmic and nuclear receptors for estrogen (ER) and progesterone (PR) throughout the estrous cycle. The ampulla showed cyclic variations in the concentration of cytoplasmic and nuclear ER, increasing during proestrus, reaching a maximum concentration during standing

estrus and declining by Days 3-4 of the cycle. The ampulla also showed changes in cytoplasmic and nuclear PR levels during the estrous cycle, with increasing concentrations found from standing estrus through the luteal phase. The isthmus had a much lower concentration of cytoplasmic and nuclear ER than the ampulla, and isthmic cytoplasmic ER showed no cyclic variation. The isthmus and ampulla showed similar levels of PRs and also had a similar expression of nuclear PR during the estrous cycle. However, no changes were noted for levels of cytoplasmic PR in the isthmus during the estrous cycle. These studies indicate that the isthmus has a constant low-level expression of cytosolic receptors, while nuclear receptor concentrations vary according to the hormonal state of the animal. The ampulla, which is believed to be more sensitized to the effects of estrogen and progesterone than the infundibulum or isthmus, revealed cyclic changes in both cytoplasmic and nuclear receptors during the estrous cycle. The higher proliferative and secretory nature of the ampulla during the preovulatory and ovulatory period may account for these differences in ER expression [Iritani et al., 1974].

Contractility and Ciliogenesis of the Oviduct

Oviductal muscular activity has been examined throughout the estrous cycle, and although investigators are not in complete agreement, it appears that the most vigorous contractions occur at about the time of estrus and ovulation. Observations done nearly 76 years ago in the pig revealed that the strength of oviductal muscular contractions was related to the dimensions of the preovulatory follicles [Seckinger 1923, Wislocki and Guttmacher 1924]. The larger the diameter of the follicle the stronger the contractions. As reviewed by Boling (1969), the predominant movement is peristaltic, and contractions occur in localized segments or loops rather than traveling long distances.

These contractions are primarily abovarian in direction. Kymographic records (radiographic examination in which the range of involuntary movements are recorded) of the pig oviduct revealed that the ampulla undergoes regular and mild contractions, while those of the isthmus are more vigorous [Kok, 1926]. Decreased contractility has been noted in the castrate rabbit although this activity did not become completely quiescent [Greenwald, 1963]. Compared to intact animals, a delay in tubal egg transport was observed in castrate animals. Greenwald (1967) showed that species differences are observed in egg transport in response to exogenous estrogen. In the rat, single injections of estrogen on Days 1, 2, and 3 of early pregnancy accelerate oviductal transport of ova [Ortiz et al., 1979, 1991]. A recent study suggests that protein synthesis is required in rat oviducts for the estrogen effect on oviductal transport to occur [Rios et al., 1997]. Several reports have indicated that estrogen and progesterone together regulate the contractility and are summarized by Boling (1969). It was observed that the oviductal musculature remains relatively quiet under the influence of estrogen, and begins to contract more vigorously when estrogen is withdrawn. Likewise, tissue under the influence of estrogen begins to contract vigorously shortly after an injection with progesterone. These data indicate that perhaps estrogen has an inhibitory effect on vigorous oviductal contractions. Much of these data appears contradictory, especially since observations at or near estrus indicate this period as having the strongest contractions. Contractions of oviductal musculature may be controlled by prostaglandins of the F series. Prostaglandin $F_{2\alpha}$ has been observed in both the Fallopian walls as well as in oviduct fluid. Lippes (1979) found that $PGF_{2\alpha}$ in human tubal fluid showed cyclical variations and had higher pre-ovulatory than post-ovulatory levels. It may be that steroid

hormones and prostaglandins program the amplitude and frequency of contractions via the regulation of calcium transport. However, information regarding control of oviductal contractions is lacking.

Ciliogenesis refers to the growth and development of oviductal cilia throughout the estrous cycle and pregnancy. Perhaps the greatest amount of information on this subject is from investigations on the number and morphology of oviductal cilia throughout the estrous and menstrual cycles and pregnancy. However, there are also several studies using experimental procedures such as hypophysectomy and ovariectomy, followed by steroid hormone replacement. As summarized by Brenner (1969), overwhelming evidence suggests that estrogen stimulates the growth of ciliated cells and progesterone antagonizes this effect. In the presence of prolonged progesterone, cells become undifferentiated and difficult to clarify [Brenner et al., 1983]. Distinct morphological changes have been noted in relation to a changing steroid hormone environment in humans [Verhage et al., 1979] and rabbits [Brenner, 1969]. Although the hormonal regulation of ciliogenesis is widely accepted, the number and character of ciliated cells varies depending on the species studied, the portion of the oviduct sampled, and the phase of the sexual cycle [Schaffer, 1908]. For example, in the domestic pig, active ciliated cells have been seen at all stages of the cycle, without any significant changes in their distribution, number, or activity [Snyder, 1923]. Only a change in cell height was observed with a maximum occurring around the time of ovulation (Day 0-3) and a minimum height about two weeks after ovulation. Observations by Buhi (unpublished data) indicate that in the pig, gradients in the number of ciliated vs secretory cells exist between the ampulla and isthmus. The pig isthmus contains about 80%

ciliated cells and 20% secretory cells (personal communication). During the luteal phase of the pig, the distal portion of the ciliated cells become constricted from the rest of the cell and are cut off in a process called deciliation [Flerko, 1954]. It has been proposed that deciliated cells fill up with secretory granules and become active secretory cells. Depleted secretory cells then become peg cells and are eventually lost at the end of the cycle [Brenner, 1969]. As summarized by Hunter (1988), ciliary beat appears to be regulated by circulating levels of ovarian hormones and is greatest at or near the time of ovulation. In most mammals examined thus far, cilia of the ampulla beat in the direction of the ampullary-isthmic junction [Blandau and Verdugo, 1976], suggesting a role for cilia in the process of egg transport to the site of fertilization.

Fertilization and Early Embryogenesis in the Oviduct

In most mammals, new life begins in the oviduct through the union of germ cells during fertilization. Wassarman (1999a) defines fertilization as the process of joining two germ cells, egg and sperm, whereby the somatic chromosome number is restored and the development of a new individual exhibiting characteristics of the species is initiated. This process includes several ordered steps: final maturation of both male and female gametes, sperm acrosome binding, acrosome reaction and penetration through the zona pellucida, fusion of sperm and oocyte membranes, the cortical granule reaction, and activation of the cell. During ovulation, fully-grown oocytes from antral follicles undergo meiotic maturation to become oocytes that are now capable of interacting with spermatozoa and being fertilized [Wassarman, 1999a]. Capacitation of spermatozoa within the oviduct is viewed as the final phase of sperm maturation, conferring upon the cells the ability to penetrate the egg investments [Hunter, 1995]. One consequence of

capacitation is an increased flagellar beat of spermatozoa, termed "hyperactivation," which is believed to assist in the penetration of the hamster zona pellucida [Yanagimachi, 1988]. A second consequence is membrane vesiculation due to point fusions on the anterior portion of the sperm head, releasing proteolytic enzymes such as acrosin [Hunter, 1995]. The acrosome reaction has been suggested to be initiated by the binding of sperm to the zona pellucida. It is generally accepted that one of the zona pellucida proteins, ZP3, is the natural agonist for stimulation of the acrosome reaction in acrosome-intact spermatozoa. Binding of spermatozoa to ZP3 activates G-proteins which stimulate a change in intracellular pH and Ca^{++} ; however, the sperm receptors that activate these G-proteins remain elusive [Wassarman, 1999b]. In the mouse, the zona matrix is composed of three ZP glycoproteins, ZP 1-3. ZP3 and ZP2 dimers are located throughout the zona pellucida and are crosslinked by ZP1 to create a three-dimensional matrix [Wassarman, 1999b]. In the pig, rabbit, and non-human primates, ZP1 has been shown to be the primary sperm receptor [Dunbar et al., 1998]. Although these proteins from different species are conserved within each family, they exhibit distinct biological properties as the mouse ZP3 is the primary sperm receptor.

Once sperm has penetrated the egg investments, its plasma membrane must bind and fuse with the oocyte vitelline membrane. Fertilin- β , a sperm membrane-bound protein, is thought to be the primary molecule responsible for binding of acrosome-reacted sperm to the plasma membrane [Frayne and Hall, 1999]. This protein is a member of the ADAM (disintegrin and metalloprotease domain) family of transmembrane proteins. The disintegrin domain of fertilin- β is believed to interact with integrins on the oocyte plasma membrane, mediating the process of fusion. Recently, it

was shown that mice that are homozygous null for fertilin- β (fertilin- β $-/-$) have a significantly reduced fertilization rate [Wassarman, 1999b]. Shortly after spermatozoa-oocyte membrane fusion, the quiescent egg "awakens" to initiate a series of biochemical events known as egg activation. This activation leads to an explosive release of Ca^{++} from intracellular storage sites, a transient increase in intracellular pH, an irreversible activation of oxidative pathways, lipid metabolism, and protein and DNA synthesis [Yanagimachi, 1988]. This activation is believed to be initiated by paternal proteins obtained during fusion. The massive release of intracellular Ca^{++} stimulates a Ca^{++} -dependent exocytosis of cortical granules (CG) which have an important role in modification of the egg envelope during fertilization. As reviewed by Yanagimachi (1988), exocytosis in mouse eggs begins near the point of sperm-egg fusion and propagates from this point in a wave-like fashion to the opposite side of the egg. The CG, which contain hydrolytic enzymes and saccharide components, alter the physical and chemical characteristics of the zona pellucida so that the zona becomes refractory to sperm attachment and penetration. This occurs due to proteolytic modifications of the sperm-binding ZP receptors. This process is referred to as the "zona reaction" and is a key event for establishing a block to polyspermy in pigs [Hunter, 1991]. Once the sperm nucleus is incorporated into the cytoplasm of the mature mouse egg (characterized by ejection of the second polar body) there is a rapid breakdown of the nuclear envelope, decondensation of chromatin, and formation of the male pronucleus.

The formation of the female and male pronucleus and their subsequent migration through the cytoplasm is due to microtubules and microfilaments of the cytosolic framework [Yanagimachi, 1988]. Besides fertilization, the oviduct is also the site of

early cleavage-stage embryonic development. Compared to other domestic species, the pig has a relatively short cleavage-stage development within the oviduct, entering the uterus at about the 4-cell stage [Kopecny, 1989]. Cleavage is invariably asynchronous in mammalian embryos cultured *in vitro* and development is retarded by about 2 h each cell cycle, thus, the development to the blastocyst stage may be an entire day longer than embryos *in vivo* [Pedersen, 1988]. This indicates that oviduct components may contribute to the morphologic and metabolic regulation of the developing embryo, leading to enhanced rates of development of *in vivo* compared to *in vitro* embryos.

Oviductal Fluid

Oviductal fluid which can be found within the tubal lumen throughout the reproductive cycle varies in volume as well as composition and is most abundant when gametes or embryos are present [Hunter, 1988]. Because tubal volume was found to be greatest during estrus and near to or just after ovulation, it was suggested that circulating ovarian steroids might regulate tubal fluid secretion. Bishop (1956) showed that after ovariectomy in estrous rabbits, tubal fluid secretion dropped from 1.2 ml to 0.2 ml/24 hr and that systemic injections of estradiol could restore fluid production to estrus volumes. Comparable observations in tubal fluid accumulation have also been noted in the ewe, cow, and pig [Hunter, 1988]. The pig was shown to have a fluid accumulation of 6.3 ml/24 hr during estrus falling to 2.1 ml/24 hr in the luteal phase [Iritani et al., 1974]. Another distinction seen between estrus and the luteal phase, is the direction of fluid flow. Seminal experiments in tubal ligation revealed significant changes in tubal fluid retention depending on the stage of the cycle. During estrus, the bulk of the fluid passes from the ampulla into the peritoneal cavity generating a retrograde flow, however, by the

time embryos descend through the uterotubal junction, much of the reduced fluid volume is being redirected toward the uterus [Hunter, 1988]. The restriction of tubal flow towards the uterus at estrus, is thought to be primarily due to edema found within the isthmic mucosa. Constriction of the isthmus is thought to be programmed by circulating levels of estrogen and wanes as a result of elevating progesterone levels [Hunter, 1988].

It is difficult to envision the relationship between tubal fluid flow and movement of the gametes through the lumen. From the observations of Parker (1931), suggestions have been made that a microflow system close to the epithelium and within the grooves or channels of the surface may differ in direction from that of the bulk flow. This microflow system may be intimately involved with the movement of spermatozoa or oocytes through the oviductal lumen. Detailed chemical analyses of oviduct fluid has revealed a complex mixture of constituents derived from plasma and the oviductal epithelium including the oviduct-specific secretory macromolecules. The passage of plasma constituents into the lumen has been referred to as 'transudation.' Because certain components in oviduct fluid are present at concentrations different from those in plasma the term transudation was expanded to 'selective transudation' [Leese, 1988]. Therefore, the term 'secretion' has been applied to the formation and discharge of *de novo* proteins from the oviductal epithelium. Protein concentration in oviductal fluid is 10-15% that found in serum [Gandolfi, 1995]. The molecules and proteins involved are transported across the epithelium within endocytic vesicles that travel from the basolateral membrane to the apical surface where their contents are released into the lumen [Parr and Parr, 1986]. The epithelial cell tight junctional complexes restrict passage of proteins into the lumen between epithelial cells. During early pregnancy in

the pig, total recovered oviductal fluid protein was greatest on days 0-3 indicating a cyclical variation in total protein [Buhi et al., 1998, Kouba unpublished]. Similar cyclic changes in total protein have been observed during the human menstrual cycle [Lippes et al., 1981].

Biosynthetic Activity

The mammalian oviduct is a highly synthetic and secretive tissue. The biosynthetic capacity has been evaluated by measuring the incorporation of radiolabeled precursor into secreted nondialyzeable macromolecules. Biosynthetic activity of the entire oviduct was greatest during proestrus, estrus, and metestrus compared to other days of the cycle in the pig [Buhi et al., 1989], sheep [Buhi et al., 1991] and cow [Malayer et al., 1988]. Synthetic differences were not observed between mated/pregnant and cyclic gilts, indicating that the presence of gametes or embryos does not affect the biosynthetic activity. These findings are in contrast to that of the rabbit where pregnant animals were shown to have decreased incorporation rates compared to non-pregnant animals [Roy et al., 1972]. The greater activity during estrus suggests that ovarian steroid hormones may regulate this function. In ovariectomized pigs [Buhi et al., 1992] or sheep [Buhi et al., 1991], biosynthetic activity was greater with estrogen than with other steroid treatments. Furthermore, pigs unilaterally ovariectomized showed no differences in biosynthetic activity from either the contra- or ipsilateral oviduct containing an ovary with follicles or corpus lutea. Therefore, circulating ovarian hormones produced by one ovary were sufficient to control protein synthesis of both oviducts [Buhi et al., 1997].

A recent study showed that the presence of a persistent dominant follicle decreased the synthetic activity of oviductal tissue in the cow [Binelli et al., 1999]. These

investigators suggest that prolonged exposure to high levels of estrogen may have caused down-regulation of the estrogen receptor, thus suppressing biosynthetic activity. Interestingly, fluctuations in the abundance of *de novo* synthesized proteins in animals containing a persistent dominant follicle, compared to a fresh dominant follicle, were observed. It was suggested by Binelli et al. (1999) that these fluctuations might be due to a release of the inhibitory actions of estrogen on certain proteins. Of the three oviduct segments, the ampulla had the greatest synthetic activity with estrogen and the lowest with progesterone. Similar to the biosynthetic capacity of the entire oviduct, the capacity of the ampulla was greatest on Days 0-4 of the estrous cycle, while the isthmus showed no differences. Likewise, the ampulla was shown to have greater biosynthetic activity than the isthmus regardless of day of the estrous cycle. Similar rates of activity were found for the ampulla and infundibulum.

Proteins, Growth Factors, and Cytokines of the Oviduct

Fluxes of molecules from the fluid bathing the serosal surface to those bathing the mucosal surface are generated giving the oviductal epithelium its physiological characteristics [Leese, 1988]. This flux includes water, electrolytes, metabolic non-electrolytes, amino acids, steroids and proteins. The secretion of specific oviduct fluid constituents will be discussed here in light of their possible contributions to gamete-embryo interactions. The majority of this section will cover the identification of proteins found within oviductal secretions, and the other constituents will be briefly highlighted. Water is not thought to be transported into the oviductal lumen through an active process, but rather in response to osmotic gradients established by the transport of ions [Leese, 1988]. Numerous ions have been identified in oviduct fluid including sodium, chloride,

potassium, bicarbonate, magnesium, calcium and inorganic phosphorous [Leese, 1988, Ellington, 1991, Boatman, 1997]. As summarized in these reviews, electrolyte concentrations in the oviduct vary considerably from those found in serum. Varying electrolyte concentrations in culture medias during *in vitro* fertilization and embryo culture have shown the importance of these constituents on fertilization and embryonic development. The volume of literature on this topic is extensive and will not be covered in this literature review. The presence of non-electrolytes in oviduct fluid has also been reviewed [see Leese, 1988, Boatman, 1997]. Metabolic precursors found in oviduct fluid include glucose, lactate, pyruvate and amino acids. Differences in concentrations of these energy substrates in oviductal fluid and blood plasma of pigs during the peri-ovulatory period have been observed [Nichol et al., 1992]. Several of these energy substrates (pyruvate and lactate) are routinely used during *in vitro* fertilization. Two amino acids, taurine and hypotaurine, have been shown to be present in oviductal fluid at concentrations much greater than those found in serum [Guerin et al., 1995]. These amino acids, as well as the converting enzyme that synthesizes hypotaurine from cysteine intermediates, such as cysteine sulphinate decarboxylase [Guerin and Menezo, 1995], have been localized to oviductal epithelial cells. Actions of taurine and hypotaurine include; osmoregulation, calcium modulation, phospholipid interactions, membrane protein receptor interactions, and antioxidation [Huxtable, 1992, Boatman, 1997]. Specific functions of these amino acids relative to the oviduct include; motility and viability of sperm, progression through the cell block, improved development of zygotes to the morulae or blastocyst stages, and increased cell numbers *in vitro* [Boatman, 1997]. The oviductal epithelium also produces another potent modulator of oxidative stress, the

antioxidant glutathione [McNutt-Scott and Harris, 1998]. Through addition of both electrolyte and non-electrolyte energy substrates and careful formulation of culture mediums to mimic oviductal concentrations of these constituents, improvements to *in vitro* fertilization and development to blastocyst have been achieved. Therefore, the relative contribution of these constituents to establishment of an appropriate oviductal environment for fertilization and early embryonic development should be underscored.

Steroids present in the oviductal environment are not generally assumed to be produced by the oviduct but arrive through serum transudation or from the ruptured follicle and cumulus cells. However, one report suggests that the oviductal epithelium contains the machinery for steroidogenesis [Ogunranti, 1992]. Many of the effects of steroids on gamete function in the oviduct are mediated indirectly through the vasculature, serosa, mucosa, and muscularis affecting protein secretion, gamete transport, and fluid accumulation [Hunter, 1988, Hafez and Blandau, 1969]. Recent evidence suggests that progesterone may have direct actions on the gametes, inducing hyperactivation and the acrosome reaction in mammalian spermatozoa [Meizel, 1995, Boatman, 1997]. Use of an anti-progesterone antibody *in vivo* showed decreased cleavage rates and embryonic cell numbers [McRae, 1994]. The actions of progesterone on spermatozoa are non-genomic in origin, and genomic actions of steroids on oviductal gametes has not been reported. Similarly, there are no reported non-genomic actions of steroids on metaphase II-oocytes through to the blastocyst stage [Boatman, 1997].

In the pig, analyses of oviductal fluid proteins reveals the presence of hundreds of proteins, most of which originate from serum as transudate [Buhi et al., 1999]. The most abundant serum proteins in oviductal fluid are albumin, heavy and light chains of

immunoglobulins and transferrin. Similar to measurements of total protein in oviduct fluid, electrophoretic analyses of serum proteins show cyclical variations in their relative distribution [Buhi et al., 1999]. Additionally, several other proteins have been localized to the oviductal epithelium and while these proteins have not been shown to be oviduct-derived and secreted, deserve some mention. Numerous protease inhibitors and enzymes have been detected including, but not limited to; alkaline phosphatase, amylase, lactate dehydrogenase, diesterase, lysozyme, acid phosphatase, esterases, lipase, aminopeptidase, glucosidase and galactosidase [Hunter, 1988]. These molecules may be important in detoxifying the oviductal environment of substrates harmful to either gametes or embryos. Recently, catalase was found in porcine, human, and bovine oviduct fluid [Lapointe et al., 1998]. Catalase concentration was greatest near estrus, and could bind to the acrosomal cap of spermatozoa. These investigators suggested that the ability of the oviduct to maintain sperm viability was partially dependent on the ability of catalase to protect sperm from oxidative damage.

Local countercurrent transfer of products from the ovary or the uterus to other parts of the reproductive tract are well known. Molecules originating from these tissues and found within oviduct epithelium or circulation include $\text{PGF}_{2\alpha}$ and PGE_2 [Harper, 1988], oxytocin [Schramm et al., 1986], and endothelin 1 [Rosselli et al., 1994]. In addition, the oxytocin receptor has been identified in the oviduct of the ewe [Ayad et al., 1990]. These molecules have been shown to be important stimulators of oviductal muscular activity. Local distributions of prostaglandin, oxytocin, and endothelin-1 in the bovine oviduct were shown to be cyclic and greater concentrations were detected at estrus in association with elevated levels of oviductal estradiol [Wijayagunawardane et

al., 1997]. In the pig, LH/hCG receptors [Gawronska et al., 1999] and receptor mRNA [Derecka et al., 1995] have been identified. It was observed that LH/hCG receptors were greatest at the preovulatory stage of the estrous cycle, and caused relaxation of the oviduct. It may be that LH in conjunction with oxytocin, endothelin 1 and prostaglandin regulate gamete/embryo transfer.

Alpha-fetoprotein (AFP) has recently been found in human oviduct fluid and may be regulated by progesterone [Lippes and Wagh, 1993]. AFP binds estrone and estradiol, acting as a carrier molecule for this steroid and its metabolites in biological fluids. AFP has also been shown to have immunosuppressive effects. Wagh and Lippes (1993) showed that AFP binds to the sperm acrosomal cap and may act as an acrosome-stabilizing factor, preventing a premature acrosome reaction. Other possible immunosuppressing agents in the oviduct are mucins. MUC1, a transmembrane mucin gene product, has been localized in the human Fallopian tube, and similar to mucins in the cervix may act as a barrier to sperm and pathogens [Gipson et al., 1997]. MUC1 may also be important for gamete and embryo transfer and prevention of adhesion to oviductal epithelium.

A large number of peptide growth factors and cytokines in the mammalian oviduct have been described [Kane et al., 1997, Buhi et al., 1997]. These factors likely originate from both the oviductal epithelium and serum transudate. Growth factors and cytokines that have been identified within oviductal fluid or the epithelium during ovulation, fertilization, and early cleavage-stage development in domestic species include; epidermal growth factor (EGF), heparin-binding EGF, transforming growth factor (TGF)- α , β , insulin-like growth factor (IGF)-I and II, IGF binding proteins (1-4),

colony stimulating factor (CSF)-1, acidic and basic fibroblast growth factor, platelet derived growth factor, granulocyte macrophage-CSF-1, interleukin-6 and the IGF-II receptor [Gandolfi, 1995, Chegini, 1996, Buhi et al., 1997, Kane et al., 1997, Buhi et al., 1999]. Several growth factor and cytokine mRNAs and proteins in the oviduct appear to be modulated by steroids and temporally associated with elevated estrogen levels at or near estrus. For examples of their presence and regulation during the estrous cycle the reader is referred to the reviews listed above. Another interesting cytokine, leukemia inhibitory factor (LIF) has been identified by the presence of mRNA and protein in the bovine [Reinhart et al., 1998] and human [Keltz et al., 1996, Barmat et al., 1997] oviduct. Reinhart et al. (1998) showed that estrogen but not progesterone, stimulated LIF synthesis and that this stimulation was not receptor-mediated, suggesting a non-genomic action for estrogen derivitized products. Retinol-binding protein (RBP) has been localized in the porcine [Harney et al., 1994], equine [McDowell et al., 1993] and ovine [Eberhardt et al., 1999] oviduct. Eberhardt et al. (1999a) showed that RBP mRNA and protein synthesis are stimulated by estrogen and that levels of RBP mRNA are greater on Day 1 of the estrous cycle than Days 5 or 10. In one experiment, embryos collected at the 1- to 4-cell stage from retinol-treated superovulated pregnant ewes and cultured *in vitro* for 7 days, showed a higher development to blastocyst (79%) than control animals (5%). Retinoids may have beneficial effects during fertilization and early embryonic development especially in embryonic morphogenesis, cell growth, and differentiation.

Oviduct-derived proteins have been identified in experiments using radiolabeled precursors for protein synthesis during oviductal organ explant or epithelial cell cultures. Electrophoretic characterization of *de novo* synthesized and secreted proteins by 1D- and

2D-SDS-PAGE and fluorography have been performed in the pig, cow, ewe, human, and baboon [Buhi et al., 1997]. However, very few of these proteins have been identified and have only been characterized in a limited fashion by isoelectric point and molecular weight. Two of these proteins, the estrogen-dependent oviduct secretory glycoprotein (OSP) and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1), have been extensively characterized [Buhi et al., 1997, 1999]. Pig OSP will be discussed in greater detail later in the literature review. TIMP-1 is a major *de novo* synthesized and secreted protein of the isthmus segment of the porcine oviduct and its mRNA and protein expression are greatest on Day 2 of the estrous cycle or early pregnancy. TIMP-1 protein production and release in the isthmus portion of the oviduct was shown to be greater with estrogen treatment indicating potential regulation of this inhibitor by ovarian steroids. This is supported by radioimmunoassay analyses of oviductal flush TIMP-1 protein, which showed significantly greater levels at Day 0 than other days of the cycle. TIMP-1, a serine protease inhibitor, has been shown to improve *in vitro* development of embryos in both the cow [Sato et al., 1994] and pig [Funahashi et al., 1997]. This evidence suggests that TIMP-1, present in the isthmus at the time of fertilization and early embryonic development, may have important regulatory actions on these processes. Recently, serine protease inhibitors of the uterus have been shown to stimulate DNA synthesis in a glandular endometrial epithelial cell line [Badinga et al., 1999], thereby suggesting a mitogenic role for TIMP-1 stimulation of embryonic development.

Four other *de novo* synthesized proteins, complement C3b, immunoglobulin A heavy chain, the carboxy terminal region of procollagen, and clusterin, have been identified in the pig oviduct by N-terminal amino acid microsequence analysis [Buhi et

al., 1998, Buhi et al., 1999b]. Similar to pOSP and TIMP-1, these four proteins appear to show synthetic gradients within the oviduct. Of the four, only clusterin and preprocollagen are found within the pig isthmus. Preliminary evidence indicates that these proteins exhibit a cyclic secretion pattern and may be partially controlled by ovarian steroids [Buhi, personal communication]. Potential functions of these four proteins include immunoprotection, lipid transport, and tissue remodeling. Of the many *de novo* synthesized proteins secreted by the porcine (>14) [Buhi et al., 1997] or bovine (>30) [Malayer et al., 1988] oviducts, only seven have been positively identified (OSP, TIMP-1, PAI-1, preprocollagen, immunoglobulin A, clusterin and complement C3b).

Oviduct Secretions and Co-culture on Fertilization and Embryonic Development

A massive amount of literature indicates that the oviduct creates a unique environment for sustaining embryo development. Prior to modern day embryo culture systems, embryos removed from the oviduct or produced as a result of *in vitro* fertilization tended to slow down or block developmentally when maintained in culture media [Bavister, 1988]. However, when embryos are transferred to the oviduct *in vivo*, development could occur from 1-cell to the blastocyst. This beneficial effect of the oviduct was shown not to be species-specific as bovine embryos could develop in the sheep or rabbit oviduct. The ability of the oviduct to overcome the cell block to development is mimicked *in vitro* by use of chemically defined media supplemented with protein additives (example; serum or BSA) and a low oxygen tension. Experiments in the cow, sheep, and mouse where embryos are transferred to oviducts *in vivo* or *in vitro* indicate an important role for this reproductive organ in sustaining embryonic growth [Bavister, 1988, Hosoi et al., 1995]. A reduced degree of embryo growth obtained in

culture media alone [Bowman and McLaren, 1970, Bavister, 1988] and growth abnormalities following embryo transfer [Farin and Farin, 1995], have been noted for embryos produced by *in vitro* fertilization.

If indeed the oviduct creates a unique environment that sustains embryo development, supplementation of oviductal fluid or the use of oviductal epithelial cell (OEC) co-cultures during *in vitro* embryo development should increase the developmental capacity. Addition of oviduct secretions/fluid to culture medium has either had no observable effect or has retarded embryo development when compared to controls [Sirard et al., 1985, Eberhardt et al., 1994]. However, careful fractionation of oviductal cell-conditioned medium and subsequent addition of these fractions during *in vitro* fertilization or embryo culture has had more promising results [Minami et al., 1992, Mermillod et al., 1993, Liu et al., 1995, Vansteenbrugge et al., 1996, Hill et al., 1996, Liu et al., 1998]. Preliminary results by these investigators have characterized, by molecular weight, the embryotrophic activities of oviductal cell-conditioned media. These activities have both high (>10 kDa) and low (<10 kDa) molecular weight components. None of the determined molecular weights correspond to OSP. Perhaps the greatest contribution of oviductal secretion analysis to date has resulted from careful evaluation of its ionic composition. The concentrations of oviductal electrolytes differs considerably from that of blood sera and media formulated to resemble the ionic composition of oviductal fluid show improved embryonic development [Bavister, 1988, Boatman, 1997]. This media, referred to as synthetic oviduct fluid (SOF), was commonly used during the late 1980s and early 1990s. However, its use is uncommon today, as laboratories are moving towards using protein-free chemically-defined culture media for repeatability.

Perhaps the most pervasive example of the contribution of the oviduct to embryo culture can be seen using OEC co-cultures. Seminal experiments by Gandolfi and Moor (1987) showed that *in vivo* fertilized sheep 1-cell embryos had a markedly increased development (8- to 16-cell stage) when co-cultured with OEC (86%) or fetal fibroblast cells (93%) than in culture media alone (13%). Interestingly, embryos cultured for 6 days in the presence of OEC had an increased development to blastocyst (46%) when compared to the fetal fibroblast cell co-culture (5%). Likewise, embryo transfer experiments, revealed that embryos cultured in the presence of OEC showed twice the viability of embryos cultured with fetal fibroblast cells. Similar results have been shown in cattle [Eyestone et al., 1987, Eyestone and First, 1989, Ellington et al., 1990]. As suggested by Bavister (1988), morphological criteria alone are not sufficient evidence of the quality of cultured embryos. Hosoi et al. (1995) designed experiments to identify the developmental stage at which embryos could be influenced by the oviductal environment. These investigators found that in both mice and cattle the oviductal influence on blastocyst development occurs during the switch from maternal to embryonic genome control. The largest contribution of OEC has been shown in humans. Human embryos co-cultured with OEC have reduced fragmentation [Bongso et al., 1989, Yeung et al., 1992, Morgan et al., 1995], better morphological characteristics [Morgan et al., 1995], higher cleavage [Morgan et al., 1995], blastulation [Bongso et al., 1989, Wiemer et al., 1993], hatching [Yeung et al., 1992] and pregnancy rates [Bongso et al., 1992]. The beneficial effect of the OEC may include the removal of toxic components from the medium and/or production of embryotrophic factors. The benefits of OEC co-cultures are not restricted to the oocyte alone but may also act on spermatozoa. Human sperm co-

cultured with bovine OEC show decreased chromatin structural degeneration [Ellington et al., 1998] and in the pig, a reduced incidence of polyspermy [Nagai and Moor, 1990, Dubuc and Sirard, 1995]. In addition to OEC effects on enhancing early embryonic development, OEC play an important role in sperm-oviduct interactions.

Sperm-Oviduct Interactions

In most domestic species, of the millions of sperm that are ejaculated during a natural mating, only a few thousand reach the isthmus of the oviduct, and there most are held in a "reservoir" [Suarez, 1998]. Of these thousand which enter the isthmus only a few reach the ampulla which is the site of fertilization. This oviductal sperm reservoir has been suggested to be involved in the prevention of polyspermic fertilization [Hunter, 1991], maintenance of sperm fertility between the onset of estrus and fertilization [Pollard et al., 1991, Chian and Sirard 1994, Smith, 1998], capacitation [Chian et al., 1995, Mahmoud and Parrish, 1996], and/or motility hyperactivation [Kervancioglu et al., 1994]. The oviductal sperm reservoir is established by spermatozoa binding to oviductal epithelial cells and has been observed in cattle, mice, hamsters, pigs, and horses [Suarez, 1998]. For most species studied thus far, spermatozoa bind to the apical membrane of isthmic oviductal epithelial cells (OEC) and adhesion is specific to the rostral region of the sperm head [Smith, 1998]. The narrowness of the isthmic oviductal lumen enhances sperm entrapment within the isthmus and increases the amount of contact between sperm and the mucosal surface. The binding moieties between spermatozoa and OEC are still unknown. Nor is it completely understood how and when sperm are released from these cells. Some evidence suggests that sperm binding and release may depend on the physical state and motor functions of the sperm. Smith and Yanagimachi (1991) reported

that hamster sperm would not bind to OEC once capacitated or hyperactivated. Likewise, hyperactivation was shown to be a primary factor for release of bound spermatozoa in the mouse [DeMott and Suarez, 1992]. Therefore, binding and release of spermatozoa may be due to changes in surface properties of the sperm head, which might lead to increased flagellar beat amplitude and asymmetry. Binding of sperm to OEC may be due to a lectin or carbohydrate present on sperm cells, as sperm attachment was inhibited by fetuin in the hamster [DeMott et al., 1995] or fucose in the cow [Lefebvre et al., 1997]. Suarez (1998) and Kervancioglu et al. (1994) suggest that epithelial secretions, initiated by signals of impending ovulation, could enhance sperm capacitation, binding, and release.

Soluble oviductal factors have been shown to induce capacitation in bull sperm [Parrish et al., 1989, Chian et al., 1995, Mahmoud and Parrish, 1996]. Heparin has been shown to induce capacitation similar to oviduct fluid [Parrish et al., 1988] and is widely used during *in vitro* fertilization of bovine oocytes. However, the intracellular signaling conditions which induce capacitation (increased intracellular Ca^{+} and pH) are not equivalent between heparin and oviduct fluid [Parrish et al., 1994]. Recently, Fazeli et al. (1999) examined induction of capacitation by porcine OEC. Similar to observations in other species, capacitated boar spermatozoa do not bind to porcine OEC. These investigators also found that unbound spermatozoa in co-culture with ampulla or isthmic OEC were slowly capacitated over time. This observation was not noted for cells of nonreproductive origin. Kervancioglu et al. (1994) reports similar observations between human OEC and vero cells (kidney epithelium). This suggests that the induction of capacitation may be specific to oviductal secretions. With boar spermatozoa, the slow induction of capacitation *in vitro* by OEC [Fazeli et al., 1999] is similar to *in vivo* bovine

sperm capacitation which may occur for as long as 24 h [Parrish, 1989]. Besides suggestions by Parrish (see above references) that a heparin-like glycosaminoglycan is responsible for capacitation activity in oviduct fluid, other protein components have been shown to induce capacitation, including the bovine estrus-associated glycoprotein [McNutt and Killian, 1991, King et al., 1994]. Capacitation can also occur spontaneously *in vitro* in a defined medium without the addition of biological fluids. This suggests that the process is intrinsically modulated by sperm itself. However, this does not rule out the influence of positive/negative regulatory factors within the oviduct. It is generally accepted that capacitation is induced by the removal of decapacitating factors, specifically cholesterol from the sperm membrane [as reviewed by Visconti and Kopf, 1998 and Cross, 1998]. Cholesterol-binding proteins are present in follicular and oviductal fluids [DeLamirande et al., 1997] and may be involved in cholesterol removal.

Structure, Regulation and Biological Actions of Plasminogen Activator Inhibitor (PAI)-1

Controlled and targeted proteolytic activity or inhibition of such activity is an important process of any biological system integrating tissue remodeling, tissue destruction, or cell migration. This extracellular proteolysis is tightly regulated by the plasminogen activator (PA)/plasmin system and inhibitors. One of the regulatory molecules, plasminogen activator inhibitor-1 (PAI-1), regulates the biological activity of PA by preventing conversion of plasminogen to the broad-spectrum enzyme, plasmin. Plasmin is primarily responsible for the proteolytic degradation of fibrin as well as other ECM proteins. Therefore, it is principally involved in fibrinolysis and breakdown of the ECM and basement membranes. PAI-1 belongs to a superfamily of "serpins" (serine protease inhibitors), which represent about 10% of the total protein in blood plasma [Gils

and Declerck, 1998]. Of these serpins, α_1 -proteinase represents about 70%. While PAI-1 has been traditionally thought of as a regulator of fibrinolysis, the plasminogen activator cascade has been shown to be important in other biological systems requiring extracellular proteolysis such as ovulation, mammary gland involution, blastocyst implantation, and tumor metastasis [Ny et al., 1993]. Plasminogen biosynthesis occurs primarily in the liver and is found in high concentrations within blood plasma (2 mM) thereby providing an unlimited source of potential proteolytic activity [Ny et al., 1993]. Thus, the rate-limiting step in these reactions are synthesis and activation of PA. The proteolytic cascade is very dynamic and must be considered a multi-component system. This multi-level system allows for precise regulation and fine-tuning at discrete focal areas requiring tissue remodeling, so that systemic proteolysis is controlled.

The primary component of this system, plasmin, is formed by proteolytic cleavage of plasminogen. One of two specific plasminogen activators, tissue-type (tPA) or urokinase (uPA), initiates the cleavage of plasminogen. Both belong to the serpin superfamily, are immunologically distinct and encoded by different genes [Ny et al., 1993]. PAI-1 binds to both tPA and uPA acting as a suicide substrate and thereby inactivating their enzymatic activity. In addition to this function, uPA- and tPA-PAI-1 complexes can bind to the multifunctional clearance receptor, low-density lipoprotein related-receptor protein/ α_2 -macroglobulin receptor (LRP/ α_2 -MR or Megalin) [Ny et al., 1993]. These complexes are internalized by endocytosis and subsequently degraded. This is another potential mechanism for PAI-1 regulation of uPA and tPA proteolytic activity. Urokinase PA also has a cell surface receptor (uPAR) along with plasminogen, and binding of these molecules to cellular surfaces is thought to lead to generation of

plasmin, which coordinates extracellular matrix (ECM) remodeling and degradation. Degradation of receptor-bound uPA is enhanced three- to four-fold following complex formation with PAI-1 [Ny et al., 1993]. Plasmin is the proteolytic enzyme primarily responsible for the degradation of fibrin (fibrinolysis), breakdown of ECM and basement membranes as well as thrombolysis. While tPA generation of plasmin is linked to the fibrinolytic activity, uPA generation of plasmin is associated with the ECM remodeling/degradation [Andreasen et al., 1990]. Plasmin can also activate promatrix metalloproteinases, procollagenase, and progelatinase. A diagram of the pericellular activation cascade for plasminogen and matrix metalloproteinases can be found in Figure 2-1.

PAI-1, a single chain glycoprotein of 50 kDa, is synthesized in an active form, which converts spontaneously to an inactive latent form that can be partially reactivated by denaturing agents such as SDS, guanidinium hydrochloride, and urea [Hekman and Loskutoff, 1985]. In plasma, PAI-1 occurs in a complex with an integrin, vitronectin, resulting in a significant stabilization [Declerck et al., 1988, Mimuro and Loskutoff, 1989]. PAI-1 forms a stable equimolar complex with both uPA and tPA, presumably of a covalent nature [Andreasen et al., 1990], and exists in at least three distinct conformational forms; active, latent, and substrate-cleaved [Lawrence et al., 1997]. Active PAI-1 decays to the latent form with a half-life of 1-2 h at 37⁰ C, unless stabilized by vitronectin. Similar to other serpins, PAI-1 has a tightly ordered tertiary structure consisting of three β -pleated sheets, A, B and C, α -helices A through I and a reactive site loop [Gils and Declerck, 1998]. The reactive site, comprising the "bait" peptide bond, is located 30-40 amino acid residues from the carboxy-terminal end. Nucleotide

sequencing of the cDNA and amino acid sequencing revealed a mature protein of 379-381 amino acids long with heterogeneity among species primarily in the amino terminus [Andreasen et al., 1990]. This protein was also found to be a glycoprotein and shares a high degree of similarity (91%) between species examined, cow, human, rat, rabbit, and mouse. Recently, pig PAI-1 was cloned and sequenced from a cDNA library prepared from cultured pig aortic cells [Bijmens et al., 1997]. The cDNA consists of a 131-bp 5' untranslated sequence, a 1206-bp open reading frame encoding a 402-amino acid protein, and a 1651-bp untranslated sequence. The preprotein is cleaved at amino acid position 23, resulting in release of the mature 379-amino acid protein, PAI-1.

Hormonal Regulation of PAI-1

PAI-1 is produced by a variety of cell lines and primary cell cultures. These *in vitro* culture systems have served as important models for obtaining information about the intracellular mechanism of action stimulated by various regulatory agents. Investigations have resulted in the identification of several hormones and steroids that control the synthesis and secretion of PAI-1. As reviewed by Andreasen et al. (1990), PAI-1 synthesis and/or activity is stimulated by glucocorticoids, insulin, endotoxin, interleukin-1, tumor necrosis factor- α , EGF, CSF-1, and phorbol esters. Inhibitors of PAI-1 synthesis and/or activity are molecules that stimulate intracellular increases in cAMP production such as follicle stimulating hormone (FSH) and leutinizing hormone (LH). Other potential regulators of PAI-1 synthesis/activity are prolactin [Liu et al., 1998] and prostaglandin $F_{2\alpha}$ [Zhang et al., 1996].

In addition to regulation by the molecules described above, PAI-1 expression and activity is regulated by ovarian steroids. Progesterone has been shown to significantly

increase PAI-1 synthesis in several endometrial cell lines [Schatz et al., 1994, Casslen et al., 1992, Casslen et al., 1995, Miyauchi et al., 1995]. As reviewed by Schatz et al. (1994), estrogen had either no effect or inhibited PAI-1 synthesis. However, the effect of progesterone on PAI-1 synthesis doubled in response to the presence of both estrogen and progesterone. Furthermore, exogenous steroids elicit similar changes in expression of PAI-1 mRNA as seen for PAI-1 protein. Progesterone was shown to not interact with the 804-bp promoter region of the human endometrial PAI-1 gene, but rather increased the stability of PAI-1 mRNA [Sandberg et al., 1997]. After menopause, hormone replacement therapy may reduce the risk of coronary heart disease that is associated with fibrinolytic activity. Women receiving estradiol were shown to have significantly lower plasma levels of PAI-1 [Lindoff et al., 1996, Shahar et al., 1996], indicating that estrogen has a significant role in inhibition of PAI-1 expression. However, some investigators suggest that estrogen might also increase PAI-1 synthesis [Sobel et al., 1995, Fujimoto et al., 1996] which is contradictory to the majority of other findings to date.

Plasminogen Activator and its Inhibitor in the Uterus and Ovary

In man and rodents, endometrial stromal cells proliferate and differentiate into decidual cells as the implanting embryo invades the endometrium in order to establish an intimate contact with the maternal blood supply. Although the implanting embryo produces matrix-degrading enzymes for implantation, ECM-degrading enzymes are also produced in the human endometrium under the control of steroid hormones [Wang et al., 1996]. In the human, an increase in tissue-factor (TF) and PAI-1, and inhibition of tPA, uPA and matrix metalloproteinases (MMPs) are associated with progestin-induced decidualization of estrogen-primed endometrial stromal cells *in vivo* and *in vitro*

[Lockwood and Schatz, 1996]. These important regulators of fibrinolysis, hemostasis, and ECM turnover in the decidualized stromal and decidual cells suggest a mechanism to explain the absence of hemorrhage during invasion of the endometrial vasculature by trophoblasts. Progesterone withdrawal reduces TF and PAI-1, yet increases uPA, tPA and MMPs thereby increasing hemorrhage, fibrinolysis, ECM degradation and vascular injury characterizing menstruation [Lockwood and Schatz, 1996]. Trophoblast cells are so highly invasive that they are called pseudomalignant and when grafted to ectopic sites, invade uncontrollably [Axelrod, 1985]. The modified uterine tissue, decidua, is rendered resistant to invasion by the well-controlled coordinate expression of protease inhibitors such as PAI-1 [Waterhouse et al., 1993, Teesalu et al., 1996, Lala and Hamilton, 1996].

The PA system has also been shown to be an important regulator of ovulation. As early as 1916, it was proposed that proteolytic activity was involved in degradation of the follicle wall at the time of ovulation [Ny et al., 1993]. Levels of plasminogen in follicular fluid are comparable to levels found in serum. Injection of agents which inhibit PA and plasmin activity into the ovarian bursa suppress ovulation [Ny et al., 1993]. Combined uPA and tPA gene-deficient mice were also shown to have a 26% reduced ovulation efficiency [Leonardsson et al., 1995]. The loss of an individual PA is functionally compensated by the activity of the remaining PA, however the loss of both is not obligatory for ovulation. As reviewed by Andreassen et al. (1990) and Ny et al. (1993), ovulation-associated hormones, FSH and LH, are effective stimulators of PA secretion from granulosa cells. In the preovulatory follicle, the LH surge stimulates a cascade of proteolytic enzymes, including PA, plasmin, and MMPs. These enzymes bring about the degradation of the perifollicular matrix and the decomposition of the

meshwork of collagen fibers which provide strength to the follicle wall [Tsafriri and Reich, 1999]. Increased ovarian proteolytic activity is controlled by high levels of PAI-1 and TIMP-1 expression in theca cells from growing follicles, ensuring their development by protection from enzymes diffusing from ovulatory follicles [Tsafriri and Reich, 1999]. FSH, LH, and activators of cAMP suppress PAI-1 synthesis and activity in granulosa cells. However, one report in the rat indicates cell-specific expression of PAI-1 and TIMP-1 mRNAs in the LH/hCG-stimulated ovary suggesting a co-expression of both enzymes and their inhibitors during ovulation [Chun et al., 1992]. PAI-1 was also shown to be a major secretory product of the corpus luteum and its expression is stimulated by PGF_{2α} [Smith et al., 1997]. These investigators suggest an important role for PAI-1 during ECM remodeling of the ovary following ovulation.

PA in the Oviduct During Fertilization and Embryo Development

A significant amount of literature has been compiled on PA activity during fertilization and its association with the preimplantation embryo. However, there are no data on its physiological inhibitor, PAI-1, in the oviductal lumen or its association with the early embryo. The importance of PAI-1 in the reproductive system is not fully understood. PAI-1 gene-deficient mice are viable, fertile, and without abnormalities in organogenesis and development [Carmeliet et al., 1993]. Other factors (PAI-2, α_2 -antiplasmin, α_2 -macroglobulin, C₁-esterase inhibitor and α_1 -antitrypsin) known to reduce plasminogen activation and/or plasmin activity, may compensate for the loss of PAI-1. However, to the authors knowledge, these proteins have not been identified in the oviduct. This portion of the discussion will concern data available on PA that may lead to insights on PAI-1 function within the oviduct.

As early as 1968, PA was shown to be associated with the oviductal mucosa [Tympanidis and Astrup, 1968]. PA activity has also been shown to be associated with rat [Liedholm and Astedt, 1975] and mouse [Sherman, 1980] preimplantation embryos from the 2-cell to blastocyst stage. Sherman (1980) showed that this activity was associated with the zona pellucida, as denuded embryos have no PA activity. These earlier studies concluded that zona-associated PA did not originate from the embryo but from genital tract secretions. The observation that PA activity steadily decreased as preimplantation development proceeded both *in vivo* and *in vitro* raises the possibility that the zona pellucida contains residual activity from follicular or oviductal fluid [Sherman, 1980].

PA activity has been associated with several important reproductive processes including oocyte maturation, fertilization, and early embryogenesis. Pig cumulus-cell oocyte complexes (COCs) have been shown to produce two PAs during *in vitro* maturation [Kim and Menino, 1995]. These PAs were shown to be tPA and tPA-inhibitor complex. Stimulators of PA activity in pig COCs, like cAMP and okadaic acid, were found to inhibit oocyte maturation. Kim and Menino (1995) suggest that although PA production is temporally associated with oocyte maturation, coordination of these two processes is differentially regulated. The production of PA in rat COCs has also been reported [Liu et al., 1986, 1987]. PA activity in rat [Salustri et al., 1985, Ny et al., 1987] and pig [Kim and Menino, 1995] COCs is through a stimulation of protein kinase A and C. Recently, cow COCs matured *in vitro*, were shown to contain mRNA for all elements of a proteolytic cascade including uPA, PAI-1, MMP-1 and TIMP-1 [Bieser et al., 1997]. These investigators suggest a potential role for this extracellular proteolysis in cumulus

expansion during oocyte maturation. In the human, transcription of PAI-1 and PAI-2 genes have been described for cumulus cells and granulosa-luteal cells [Piquette et al., 1993]. Similarly, cow COCs show production of PA during *in vitro* maturation and this production was stimulated by EGF [Park et al., 1999]. This activity was shown to be associated with uPA and was not present in denuded oocytes, indicating the importance that cumulus cells play in its synthesis. For a current discussion on the production of PA in COCs relative to the time of germinal vesicle breakdown and resumption of meiosis, the reader is referred to Park et al. (1999).

As reviewed by Huarte et al. (1993) the PA/plasmin proteolytic cascade may have a significant role during fertilization. Proteolytic enzymes participate in multiple phases of mammalian fertilization including the acrosome reaction, sperm-binding to the zona pellucida, zona pellucida penetration, and the zona reaction. Mouse gametes express plasminogen-dependent proteolytic activities; ovulated eggs synthesize and secrete tPA while ejaculated spermatozoa have uPA activity. Likewise, plasminogen was shown to bind to both mouse spermatozoa and eggs, and the presence of plasminogen increased the *in vitro* fertilization rate [Huarte et al., 1993] presumably due to increased plasmin generation. In addition, antibodies, which inhibit the catalytic activity of plasmin, were shown to decrease the fertilization rate. Exogenously added plasminogen was likely converted to plasmin by zona pellucida-associated uPA or tPA activity. These findings are supported by previous work indicating that a positive correlation existed between the ability of oocytes fertilized *in vitro* and the PA activity of the corresponding follicular fluid and granulosa cells [Deutinger et al., 1988, Milwidsky et al., 1989]. Zhang et al. (1992) showed that tPA was released from rat oocytes as a result of oocyte activation and

suggested that tPA may act in the perivitelline space on the zona pellucida during fertilization and/or activation. As described by Zhang et al. (1992), tPA may be a component of cortical granules that are involved in the zona block to polyspermy. In their study, activation-induced zona hardening (limited proteolysis of zona protein ZP2) was prevented by a anti-tPA monoclonal antibody. Inadvertent activation under *in vitro* conditions in mouse and human IVF systems often times leads to premature loss of fertility in respective oocytes. Addition of leupeptin, a serine protease inhibitor, during mouse oocyte calcium-ionophore activation reversed the decrease in the capacity of oocytes to fertilize and develop *in vitro* [Tawia and Lopata, 1992]. The data support evidence for PA activity in zona hardening and suggests a coordinate expression between activator and inhibitor in regulating this process. Additionally, supplementation of culture medium with proteases increased hatching rate of mouse embryos [Lee et al., 1997], while addition of protease inhibitors to culture medium inhibited hatching *in vitro* [Dabich, 1981, Yamazaki et al., 1985]. This suggests an important role for oviductal-derived protease inhibitors in the prevention of premature hatching, prior to the correct developmental stage within the uterus. The effect may be directed towards proteolysis of the zona matrix. Zona of porcine oviductal oocytes and embryos were shown to be more resistant to proteolytic digestion than either follicular oocytes or embryos recovered from the uterus [Broermann et al., 1989], indicating an important role for protease inhibitors in protecting the zona pellucida from degradative proteases.

Preimplantation mouse [Harvey et al., 1995, Zhang et al., 1996], rat [Zhang et al., 1994], sheep [Menino et al., 1989, Bartlett and Menino, 1993] and cow [Dyk and Menino, 1991, Berg and Menino, 1992] embryos were shown to have PA activity.

Kaekuahiwi and Menino (1990), showed that as embryonic size and cell number increase and development progresses, bovine embryos liberate more PA. Recently, bovine embryonic PA was shown to induce changes in the electrophoretic protein profile of the zona pellucida when embryos were incubated with exogenous plasminogen [Cannon and Menino, 1998].

In addition to PA's association with maturation, fertilization and embryogenesis, it might also have an important role in spermatozoa physiology. Urokinase PA is synthesized in epithelial cells of the caudal epididymis, vas deferens, and seminal vesicles [Huarte et al., 1987], while tPA was found in the prostate gland [Reese et al., 1988]. In addition, Sertoli cells have been shown to secrete PA and PAI-1 [reviewed by Gilabert et al., 1995]. The presence of uPA and tPA has been shown in ejaculated spermatozoa of man and various animal species [Smokovitis et al., 1987]. These investigators report a direct influence on sperm motility and suggest that the PA are membrane bound. Recently, boar spermatozoa were shown to have uPA and tPA associated with spermatozoal membranes and differences were detected in their localization [Smokovitis et al., 1992]. The outer acrosomal membranes contained tPA while the inner acrosomal membrane contained both tPA and uPA.

Structure, Regulation, and Biological Actions of Oviductal Secretory Glycoprotein (OSP)

In vitro Synthesis and Hormonal Regulation of OSP

Experiments by Oliphant and Ross (1982) in the rabbit describe the identification and purification of three sulfated glycoproteins from oviduct fluid. This investigation was one of the first studies utilizing a radiolabeled precursor, [^{35}S], to evaluate incorporation into macromolecular components of oviductal epithelial cells and oviductal

explant tissue in culture. Utilizing this technique, Oliphant and Ross (1982) reported the *de novo* synthesis and secretion of glycoproteins by oviductal epithelium. Subsequently, numerous studies have been done in various species. Evaluations of stage-specific proteins in the pig oviduct were first reported by Buhi et al. (1989). The *de novo* synthesis of porcine oviductal proteins by cyclic and early pregnant tissues in explant culture revealed that incorporation of [³H]-leucine into nondialyzeable macromolecules was greatest on Days 0 and 2 and secretory activity was lowest on Days 10 to 15. The ampulla was shown to have greater incorporation than the isthmus and increased rates of incorporation were temporally associated with elevated estrogen at proestrus, estrus, and metestrus [Buhi et al., 1989]. 1D-SDS-PAGE and fluorographic analysis of oviductal culture medium revealed three proteins of 335 k, 115 k, and 85 k M_r, associated with proestrus, estrus, and metestrus. High resolution 2D-SDS-PAGE further resolved the isoelectric and molecular weight variants of these radiolabeled proteins initially detected by 1D-SDS-PAGE [Buhi et al., 1990]. The major 115 k M_r band was resolved into two major glycoproteins, one basic (100 k M_r, pI > 8) and one acidic (100 k M_r, pI 4.5-5.5). The 85 k M_r protein was resolved into a very acidic (pI < 4) protein of 75 to 85 k M_r. These three proteins have been designated pOSP E1 (85 k M_r), pOSP E2 (100 k M_r; acidic) and pOSP E3 (100 k M_r; basic). It was suggested by Buhi et al. (1990) that these denatured and reduced subunits may be part of a higher molecular weight complex and were related (pOSP E2 had the same N-terminal sequence as E1 and the N-terminal sequence for E3 was identical to an internal sequence of E2). Incorporation of [³⁵S]-glucosamine indicates that these three estrus-associated proteins are glycosylated [Buhi et al., 1990].

Oviduct-specific secretory proteins have been identified in human [Verhage, 1988, Buhi et al., 1989], baboon [Fazleabas and Verhage, 1986, Verhage and Fazleabas, 1988], cow [Malayer et al., 1988, Boice et al., 1990], sheep [Sutton et al., 1984, Sutton et al., 1986, Gandolfi et al., 1989, Buhi et al., 1991, Murray, 1992], mouse [Kapur and Johnson, 1985] rhesus monkey [Verhage et al., 1997], and hamster [Robitaille et al., 1988, Abe et al., 1998]. Although isoelectric points are similar for the subunits examined in other species, differences have been noted in molecular weights for these glycoproteins. Studies on these glycoproteins have led to several key observations. First, these glycoproteins are primarily secretory products of the ampulla, although their synthesis can be seen in other segments at a much lower rate [Verhage and Fazleabas, 1988, Buhi et al., 1990, Boice et al., 1990, Gandolfi et al., 1991, Murray, 1992, and O'Day-Bowman et al., 1995]. Second, the presence of these proteins only during estrus, temporally elevated at estrus or the follicular stage in humans indicates that this protein is regulated by estrogen. Therefore, the pattern (but not presence) of distribution for these proteins is dependent on the presence or absence of specific ovarian steroids. In the pig, treatment of ovariectomized (OVX) gilts with estrogen increased incorporation rate in the ampulla and revealed that the oviduct-specific glycoproteins were estrogen-dependent [Buhi et al., 1992]. This is supported by the observation that progesterone antagonizes/abrogates the stimulatory effect of estrogen on their synthesis. Similar observations on OSP steroid regulation using OVX animals have been made in sheep [Buhi et al., 1991, Murray and DeSouza, 1995] and baboon [Verhage and Fazleabas, 1988]. Newborn golden hamsters (1.5 days) injected daily with estrogen or progesterone showed that estrogen could induce hamster OSP synthesis in the undifferentiated

epithelial cells of neonates, while progesterone could not [Abe et al., 1998]. This family of estrogen-dependent and oviduct-specific glycoproteins is the most abundant radiolabeled secretory product of the oviduct, yet assigning specific functions to these proteins has been elusive. Investigations on the localization of OSPs in the oviduct and their association with the ovulated oocyte and early embryo, as detailed below, suggest possible involvement during fertilization and early cleavage-stage embryonic development.

Immunolocalization of pOSP in the Oviduct, Oocyte, Spermatozoa and Early Embryo

Immunogold localization of pOSP in the pig has been evaluated at the cellular level utilizing electron microscopy [Buhi et al., 1993]. These investigators found that pOSP was localized to putative secretory granules in non-ciliated secretory cells of the ampulla from both cyclic and OVX estrogen-treated gilts. This study was not extended to evaluate distribution of pOSP in either the infundibulum or isthmus of the pig. Similar observations of OSP localizing to secretory granules in the ampulla have been observed in sheep [Murray, 1992, Gandolfi et al., 1991], cow [Boice et al., 1990], baboon [Verhage et al., 1990, Verhage et al., 1989], rhesus monkey [Verhage et al., 1997], and human [O'Day-Bowman et al., 1995, Rapisarda et al., 1993]. Studies done in the human and baboon identified OSP in secretory granules of the isthmus as well as the ampulla, indicating that this segment also synthesizes OSP. Evaluations of colloidal gold densities in segments treated with estrogen, progesterone, or estrogen + progesterone have not been performed. Therefore, it is unknown whether there are dynamic changes of OSP in secretory granules relative to hormonal status, except for the observation with estrogen alone, as detailed above. Hamster OSP of oviductal origin is associated with uterine

epithelial cells during the first 3 days of pregnancy, but is reduced by Day 4, and is absent by Days 5 and 6 [Roux et al., 1997]. Because Days 5 and 6 correspond to the time of implantation in the hamster, and levels of OSP remain constant in the oviduct, these investigators suggest that OSP may be involved in uterine receptivity.

The macromolecular composition of the zona pellucida in the mammalian oocyte/embryo is quite complex and three major glycoproteins have been identified each containing about 15 isoelectric species [Dunbar, 1983]. Several studies have revealed changes in the composition of the porcine zona pellucida during development from the oocyte to the 4-cell embryo. Brown and Cheng (1986) observed that the zona pellucida of the follicular oocyte to the early embryo acquired three glycoproteins from the oviduct at estrus. These investigators observed two proteins of 250 k and 90 k M_r when the zona pellucida was analyzed by 1D- and 2D-SDS-PAGE under non-reducing conditions. When these proteins were electrophoresed under reducing conditions, subunits of 90 k, 79 k, and 69 k M_r were detected. Similar differences in the macromolecular composition of zona pellucida from pig oocytes, eggs, and zygotes have been observed by Hedrick et al. (1987), although there is some discrepancy in the molecular weight of these proteins when compared to those of Brown and Cheng. These are the first studies indicating that proteins of oviductal origin associate with the zona pellucida and that their molecular weight (80-90 k) corresponds to that of pOSP. The study by Buhi et al. (1993) supports these earlier results and specifically indicates that oviductal pOSP associates with unfertilized oviductal oocytes and early embryos. Pig OSP immunoreactivity was observed through early development to Day 7 hatched porcine embryos but had disappeared by Day 9 of pregnancy [Buhi et al., 1993]. In addition to association with the

zona pellucida, pOSP also associates with flocculent material in the perivitelline space and with vitelline and blastomere membranes. Limited data supports the possibility that the embryo itself endocytoses OSP during its early development. Hamster OSP has been localized in coated pits, apical vesicles, multivesicular bodies, and lysosome-like structures [Kan and Roux, 1995]. The association of pOSP with the early developing embryo suggests this protein may have an important role during fertilization and early cleavage-stage embryonic development.

Localization of OSP to the zona pellucida of oviductal oocytes or early embryos has been shown in the hamster [Kan et al., 1989, Kan and Roux, 1995, Abe and Oikawa, 1990, Leveille et al., 1987], cow [Wegner and Killian, 1991], sheep [Gandolfi et al., 1991], rhesus monkey [Verhage et al., 1997], and baboon [Boice et al., 1990]. In the mouse, OSP was not associated with the zona pellucida and was only localized within the perivitelline space [Kapur and Johnson, 1985]. An interesting observation by Kan et al. (1995) was that hamster OSP localized to the flocculent material in the perivitelline space in oviductal oocytes only after fertilization. These investigators suggest that OSP interacts with secretions of cortical granules after fertilization and may be involved in the block to polyspermy. This hypothesis is supported by our work on polyspermy described in Chapter 4.

While observations for an association of OSP with the oviductal oocyte or early embryo are consistent across species studied thus far, a similar consensus does not exist for localization of OSP to spermatozoa. In the hamster [Kimura et al., 1994, Boatman and Magnoni, 1995] and cow [King and Killian, 1994, Abe et al., 1995], OSP has been shown to associate with the spermatozoal membrane. However, a report in the human

[Reuter et al., 1994] failed to find an association of human OSP with human spermatozoa.

Molecular Biology of pOSP

Cloning and characterization of a full length cDNA for pOSP has been reported by Buhi et al. (1996). A full length clone (2022-bp) was identified and sequenced, yielding an open reading frame of 1581 bp that coded for a protein of 527 amino acids. A putative signal sequence was identified (a.a. 1-21) and is highly conserved, having 100% homology among bovine, human, and ovine signal sequences. Sequence analysis of pOSP revealed 3 consensus N-glycosylation sites as well as several potential O-glycosylation sites, suggesting large amounts of carbohydrate addition. This would also explain the differences between the predicted native molecular weight of 55,600 and those observed by 2D-SDS-PAGE (E1; 75 k, E2,3; 100 k M_r). The pOSP cDNA sequence revealed significant identities and similarity to oviductal secretory glycoproteins (OSP) from a number of other species, especially in the 5' end of the open reading frame. Sequence analysis of pOSP cDNA also revealed two potential phosphorylation sites, a consensus heparin-binding sequence, a region similar to the chitinase catalytic site, and a potential C-terminal region similar to a chitinase binding domain. However, this protein does not have chitinase activity and is missing an essential amino acid required for such activity. Observations have been made establishing a structural similarity between OSPs and the chitinase protein family [Arias et al., 1994, Sendai et al., 1995, Suzuki et al., 1995, Buhi et al., 1996]. Sendai et al. (1995) has suggested that the chitinase-like structure in OSPs may be involved with carbohydrate moieties of the oocyte or surface of the spermatozoa. Since chitinase binds

to chitin (poly- β 1,4-N-GlcNAc) and hydrolyzes it, Suzuki et al. (1995) suggests that OSPs may be a GlcNAc binding protein, which interacts with carbohydrate components, located on the zona pellucida. OSPs have been cloned in several other species including the baboon [Donnelly et al., 1991], human [Arias et al., 1994], hamster [Suzuki et al., 1995], mouse [Sendai et al., 1995], cow [Sendai et al., 1994], sheep [DeSouza and Murray, 1995] and rhesus monkey [Verhage et al., 1997]. These researchers also found that a significant degree of homology exists among oviduct-specific secretory glycoproteins of various mammalian species studied thus far. However, OSP cDNA appears to lack homology to the frog oviduct-specific protein-1 gene found in *Xenopus* oviduct cells [Donnelly et al., 1991]. Sequence analysis of the various species listed above revealed several N- and O-glycosylation sites, which may reflect why differences are observed between species in isoelectric point and molecular weight when evaluated by 2D-SDS-PAGE. Identification of the polypeptide precursors and characterization of their biosynthetic maturation within oviductal cells for hamster OSP has been examined in detail and hypothesis on the polymorphism of these variants due to translational and posttranslational events has been reviewed by Malette et al. (1995). Southern blot analysis of genomic DNA has shown that there is a single copy of the hamster OSP gene in the hamster genome and PCR amplification revealed that it is contained within a single exon, excluding the possibility of alternative splicing [Paquette et al., 1995]. Although these studies have not been done in other species to date, immunological and northern blot analysis suggest that the family of OSPs detected by 1- and 2D-SDS-PAGE all derive from a single gene. Hamster OSP has recently been referred to as a secretory mucin, due to extensive O-glycosylation (> 50%) not seen in other species, tandemly

repeated amino acid motifs, and the presence of multiple alleles (Paquette et al., 1995). These tandem repeats are present in rodents and humans but are not seen in ungulates, raising interesting questions from an evolutionary viewpoint. A review of the various sequences cloned revealed significant differences in the C-terminal region of the OSPs and may reflect species differences in the OSP molecule. For instance, Sendai et al. (1995) identified a unique seven-residue repeat sequence (21 repeats) in the C-terminal side of mouse OSP which are not seen in cow, sheep, pig or baboon OSP.

Probes for pOSP mRNA detected only a single 2.25 kb message in oviductal tissue and mRNA was present in all 3 segments of the oviduct. Levels of mRNA were greatest in the ampulla indicating this segment as the primary site of its expression. Northern blot analysis of numerous other tissues (heart, intestine, cervix, kidney, endometrium, myometrium, spleen, lung, aorta, liver, or stomach) confirmed that expression of this protein is specific to the porcine oviduct. Steady-state levels of pOSP mRNA in the oviduct were greatest at estrus (Days 0,1) consistent with elevated estrogen, decreased rapidly by Day 2, and remained low throughout diestrus. These data correlates well with previous reports on pOSP protein synthesis [Buhi et al., 1990, Buhi et al., 1992]. Levels of pOSP mRNA in oviducts of steroid-treated OVX gilts confirmed that this protein's expression is up-regulated by estrogen and that progesterone antagonizes this effect. Data from other species including human [Arias et al., 1994], baboon [Donnelly et al., 1991], and bovine [Sendai et al., 1994] have also shown that the message for this protein is greatest during the late follicular phase and in estradiol-dominated oviducts.

A study in the hamster, utilizing *in situ* hybridization for detection of OSP message, found evidence for OSP in both the ampulla and isthmus, although the signal intensity was greatest in the ampulla [Komiya et al., 1996]. The distribution of OSP message was different within the two sections, with the ampulla having signal detected in both the perinuclear and basal regions, while the isthmus only had signal in the basal compartment of epithelial cells. Komiya et al. (1996) also found that the message for OSP is greatest when serum estradiol/progesterone levels are higher and that the mRNA for OSP wanes with age and correlates with decreasing serum estradiol levels. However, these investigators also showed that progesterone is required for hamster OSP gene expression, unlike data from the baboon [Donnelly KM et al, 1991] and pig [Buhi et al., 1996], which shows OSP strongly suppressed by progesterone. Another interesting difference is that the hamster [Paquette et al., 1995] rabbit [Donnelly et al., 1991], and mouse [Donnelly et al., 1991] showed a constant level of mRNA expression for OSP throughout the estrous cycle which differs from that of other species observed thus far where a strong estrogen-dependence is required for its expression. Contrary to findings by Komiya et al. (1996), Murray and DeSouza (1995) found that transcripts encoding sheep OSP were in the basal compartment and at the apical tips in fimbria and ampulla epithelial cells at the free margins of mucosal folds. Because mRNA was localized to the apical tips, these investigators suggested that mRNA encoding OSP was translated at a unique cytoplasmic foci. Electron microscopy reveals that all the cytoplasmic machinery required for protein synthesis is also located within the apical tips of oviductal epithelial cells, which suggests a quick response time from the point of translation to release of OSP into the lumen [Murray and DeSouza, 1995].

Cyclical, structural, and functional changes in the oviduct are thought to be brought about primarily by the actions of estradiol and progesterone [Verhage and Jaffe, 1986], and numerous reports have been cited above showing regulation of OSPs and pOSP by estrogen/progesterone levels. Recent studies have demonstrated that human [Lie et al, 1993] and pig [Gawronska et al., 1999] oviducts contain luteinizing hormone (LH)/human chorionic gonadotropin (hCG) receptors, suggesting that factors other than circulating ovarian steroids may regulate oviductal function. One study by Sun et al. (1997), revealed that culturing bovine oviductal epithelial cells with hCG resulted in a time and dose-dependent increase of OSP protein and transcript. A nuclear transcription run-on assay was used to determine that hCG did not increase the transcription rate of the gene but rather "stabilized" its transcript [Sun et al., 1997]. These investigators suggested that higher LH levels present during the periovulatory periods may act to stabilize the message for OSP in the oviduct, thus leading to an increased secretion rate. Recently, heterozygous mutant mice have been generated, which lack a large portion of the OSP coding region [Sendai et al., 1999]. In the future, homozygous offspring may clarify the role of this protein *in vivo*.

Biological Actions of OSPs

The observations that in all species examined so far, with the notable exception of the mouse, OSPs associate with the zona pellucida, suggest an intimate role for these estrogen-dependent proteins during fertilization and early cleavage-stage embryonic development. However, the abundant information detailing a morphological association of OSPs with spermatozoa, oocytes, or embryos does not correspond to a suitable amount of functional information. Functional information regarding OSPs have been hindered by

difficulties in the protein purification process. In fact, no direct *in vivo* data on precise functions of these proteins are available to date. However, several *in vitro* observations of activities associated with the inclusion of OSP have led to some attractive hypotheses.

Macromolecules secreted by the hamster oviduct in the ampulla region have been shown to facilitate penetration of sperm through the egg investments [Boatman et al., 1994] and follicular eggs collected from the ampulla were shown to be more penetrable and fertilizeable than follicular eggs collected from the ovarian bursa or unovulated follicles [Boatman and Magnoni, 1995]. Similar increases in penetration rates have been observed in the bovine [Martus et al., 1998] and were linked to the addition of concentrated bovine OSP during *in vitro* fertilization (IVF). One conflicting report in the hamster [Kimura et al., 1994] indicates that penetration rates were decreased in the presence of OSP. These data, although partially conflicting, indicate that association of OSP with the oocyte may have a functional role during fertilization. It is unknown whether these observed effects were due to actions on spermatozoa, oocyte or both. Martus et al. (1997) showed that exposure of bovine oocytes to bovine OSP during any phase of the IVF process increased fertilization rates, while exposure of spermatozoa to bovine OSP had no effect.

Several investigators suggest that OSPs induce capacitation [McNutt et al., 1992, Anderson and Killian 1994] and/or the acrosome reaction. Therefore, an increase in the number of capacitated or acrosome-reacted spermatozoa could possibly lead to the above observations on increased penetration. However, direct evidence for the estrogen-dependent OSP on these activities is lacking.

Another potential modulator for this increase in penetration may be due to actions on spermatozoa movement characteristics. Abe et al. (1995) found that bovine OSP effectively maintained the viability and motility of bovine spermatozoa relative to control medium and that this activity was dose-dependent. The ability of bovine OSP to maintain motility and viability may allow a greater proportion of spermatozoa to penetrate the egg investments and subsequently fertilize the oocyte. The OSPs may also have a role in gamete recognition and binding. In the hamster [Schmidt et al., 1997a, 1997b] and human [O'Day-Bowman et al., 1996], OSP was found to increase the number of tightly bound sperm attached to the zona pellucida. This increase might also lead to the increased penetration rates described above. These data are supported by the observation that a redistribution in the localization of hamster OSP occurs depending on the state of capacitation [Boatman and Magnoni, 1995].

Functional data on embryonic development are especially lacking, and investigations to date are less than encouraging. No effects of bovine OSP [Vansteenbrugge et al., 1997] or ovine OSP [Hill et al., 1997] were observed on bovine embryonic development. Numerous investigations have shown the need for homologous systems when studying this protein, therefore observations by Hill et al. (1997) may be due to the heterologous system employed. Previous studies by Hill et al. (1996 a, b) examining ovine OSP effects on ovine embryonic development demonstrated several subtle but consistent results. The most significant of these being that a reduced proportion of one-cell embryos underwent first cleavage, but no overall decrease on the proportion of blastocysts that formed. These investigators suggest that this may reflect a selection mechanism occurring *in vivo*. Bovine OSP has been found to increase the

number of bovine zygotes that cleaved compared to controls, but by Day 7 of embryonic development, no difference could be detected in the number of blastocysts that formed [Martus et al., 1997]. However these investigators did observe an increased number of blastocysts on Day 6 of *in vitro* culture. This may suggest that bovine OSP, while not increasing the number of blastocysts, increases their rate of development. However, a slight loss in the number of blastocysts between Day 6 and Day 7 was shown and might indicate that the concentration of bovine OSP tested or length of exposure (>6 days) is detrimental to subsequent development. This increased rate of development may be due to an unknown stimulation on the expression of maternal or embryonic genes and increased protein synthesis. One study indicates that *in vivo* fertilized pig embryos (one- or two-cell stage), cultured *in vitro* in the presence of one subunit (97 k M_r) of semi-purified pOSP, showed increased rates of incorporation of methionine into protein at the four-cell stage [Wallenhaupt et al., 1996]. The data described above indicate that pOSP may facilitate or enhance fertilization and early cleavage-stage embryonic development.

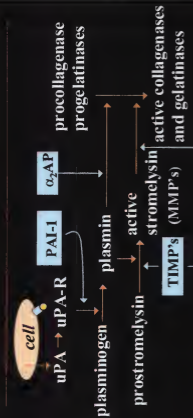
This review thus leaves a number of unanswered questions which need to be addressed. The first is; what are the unidentified *de novo* synthesized and secreted macromolecules of the oviduct? The second is; for those proteins which have been identified and characterized, what is the specific function(s) in relation to reproductive processes in the oviduct; union of gametes, fertilization, and early cleavage-stage embryonic development. Chapters 3, 4, and 5 describe the identification, characterization, and regulation of PAI-1 in the oviduct. The objectives of these three chapters include; 1) localization of PAI-1 within the oviduct, 2) hormonal regulation of oviductal PAI-1 by ovarian steroids, 3) expression of PAI-1 mRNA and protein during

early pregnancy, 4) evaluation of PAI-1 and uPA activity in the oviduct during early pregnancy, and 5) localization of PAI-1 on oviductal oocytes and embryos. Chapter 6 will examine the functionality of pOSP during in vitro fertilization and embryo culture. Effects of pOSP on the fertilization rate, polyspermy rate, and development to blastocyst will be examined. Results of these studies will begin to define the roles of oviductal PAI-1 and pOSP during fertilization and embryonic development.

Figure 2-1. Pericellular activation cascade for plasminogen and matrix metalloproteinase.

uPA, urokinase plasminogen activator; uPA-R, urokinase plasminogen activator receptor; PAI-1, plasminogen activator inhibitor-1; α_2 AP, alpha-two anti-plasmin inhibitor; TIMP, tissue inhibitor of matrix metalloproteinase; MMP, matrix metalloproteinase. Proteolytic enzymes and inhibitors (outlined in blue) involved in the regulation of fibrinolysis and extracellular matrix remodeling/degradation.

Pericellular activation cascade for plasminogen and matrix metalloproteinases



CHAPTER 3

IDENTIFICATION AND LOCALIZATION OF PLASMINOGEN ACTIVATOR INHIBITOR-1 (PAI-1) IN THE PORCINE OVIDUCT

Introduction

The porcine oviduct provides an important microenvironment for final maturation of gametes, fertilization, and early cleavage-stage embryonic development. In part to provide an effective environment for these reproductive processes, numerous proteins derived from serum as a transudate or from oviductal epithelium contribute to the composition of oviductal luminal fluid [Feigelson and Kay, 1972, Sutton et al., 1984, Buhi et al., 1997]. Oviductal luminal fluid and its protein composition vary during the estrous cycle and early pregnancy, and maximal levels of oviductal fluid appear to coincide with elevated estrogen [Hunter, 1988]. When estrogen is maximal, Days 0 and 1 of the estrous cycle or early pregnancy, biosynthetic activity in the oviduct is greatest [Buhi et al., 1989]. The infundibulum and ampulla, regardless of day of the estrous cycle or early pregnancy, have a biosynthetic activity significantly greater (2-3 times) than that of the isthmus [Buhi et al., 1997]. This activity, measured in explant culture-conditioned media, reflects the *de novo* synthesis and secretion of secretory proteins from these three segments.

The relative importance of oviductal-derived proteins to fertilization and early embryonic development is not known, nor have many of these proteins been identified. Several studies have shown the importance of utilizing the oviduct or its constituents for

enhancing embryo development *in vitro* [Archibong et al., 1989, White et al., 1989, Liu et al., 1998]. In the pig, glycoproteins derived from oviductal fluid have been shown to associate with the zona pellucida of oocytes after ovulation, at fertilization and during early embryonic development [Brown and Cheng, 1986, Hedrick et al., 1987, Buhi et al., 1993]. It has been suggested that these proteins may be facilitating the beneficial effects of oviductal epithelial cell co-cultures on embryo development *in vitro* [Buhi et al., 1997].

The pig oviduct has been shown to synthesize and secrete *de novo* 14 major proteins into explant culture medium [Buhi et al., 1990]. The majority of these proteins have been described electrophoretically by isoelectric point and relative molecular mass. Two of these proteins have been identified and characterized, the porcine oviduct-specific secretory glycoprotein (pOSP) [Buhi et al., 1992] and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) [Buhi et al., 1996]. Of the numerous proteins yet to be identified, one protein with an apparent molecular weight of 45,000, appeared to be synthesized and distributed similar to TIMP-1. Like TIMP-1, this protein was shown to incorporate ^3H -glucosamine, be composed of 5-6 isoelectric species, and be synthesized primarily by the isthmus [Buhi et al., 1990]. Thus, with characteristics similar to oviductal TIMP-1, it was suggested that the 45,000 M_r protein may also be a protease inhibitor.

This study was designed to further identify and characterize the 45,000 M_r *de novo* synthesized protein of the porcine oviduct and to better understand specific protein contributions of each oviductal segment relative to ovulation, gamete transport, fertilization and early cleavage-stage embryonic development. With identification of the

unknown 45,000 M_r protein as porcine plasminogen activator inhibitor (PAI)-1 (described here in Chapter 3), studies were designed to examine its synthesis within the infundibulum, ampulla, and isthmus, and to evaluate its distribution throughout the oviduct. While PAI-1 has been localized in the uterus and ovary from a variety of species and potential mechanisms examined, no studies to our knowledge have been done to assess PAI-1 within the oviduct.

Materials and Methods

Materials

Acrylamide, N,N' diallyltartardiamide, urea, Nonidet P-40, and sodium dodecyl sulfate were purchased from Gallard-Schlesinger (Carle Place, NY); X-Omat AR film and photography reagents were a product of Eastman Kodak Co. (Rochester, NY); amino acids and protein standards were purchased from Sigma-Aldrich (St. Louis, MO); ampholines were from Pharmacia-Biotech (Piscataway, NJ); all other supplies and reagents for gel electrophoresis were purchased from Bio-Rad Laboratories (Richmond, CA) or Fisher Scientific (Orlando, FL). All medium and culture supplies were purchased from Life Technologies (Grand Island, NY). L-[4,5- 3H]leucine (specific activity, 120 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). Immobilon-P (PVDF) membranes were purchased from Millipore Corporation (Bedford, MA); Vectastain ABC Elite kit was obtained from Vector Laboratories (Burlingame, CA). Affinity-purified goat anti-human PAI-1 was purchased from American Diagnostica (Greenwich, CT); goat IgG and normal rabbit serum (NRS) were purchased from Sigma-Aldrich. All other reagents, including column chromatography supplies, were products from Sigma, Fisher, Life Technologies or Pharmacia-Biotech.

Tissue Collection, Explant Culture, and Electrophoresis

Florida crossbred (Yorkshire x Duroc x Hampshire) or European Large White gilts were observed daily for behavioral estrus in the presence of an intact boar. After the completion of at least two estrous cycles, animals for pregnancy studies were bred on the first day of standing estrus, designated as Day 0, and 24 h later (except for gilts assigned to Day 0). Pregnant or cyclic gilts were taken to the abattoir on Days 0, 2, and 12 for slaughter. Oviducts were collected aseptically and separated by gross dissection into the three functional segments, which were then cultured as previously described [Buhi et al., 1989]. For culture, 0.5 g of tissue was incubated in leucine-deficient (modified) Eagle's minimum essential medium (MEM) containing 100 μCi [^3H]-leucine for 24 h at 39 $^{\circ}\text{C}$ in a defined atmosphere. Conditioned culture media was then separated from tissue and frozen at -20 $^{\circ}\text{C}$ until analyzed by 2D-SDS-PAGE and fluorography or utilized for purification of the 45,000 M_r protein.

In order to evaluate synthesis of the 45,000 M_r protein within the three segments, oviductal tissue was collected from Day 2 pregnant Large White gilts and cultured as described above. Culture media was dialyzed (12,000 molecular weight cut-off) against 10 mM Tris-HCl buffer (pH 7.6), containing 0.15 M NaCl and 0.02% (w/v) NaN_3 , followed by dialysis against dH_2O (2 changes, 4L each, 24 h each) at 4 $^{\circ}\text{C}$. Total protein content of dialyzed culture media was measured by the Bio-Rad microassay (according to manufacturer's instructions) and radiolabeled proteins measured by liquid scintillation spectrophotometry. Samples representing each oviductal segment, containing 100,000 cpm, were lyophilized, solubilized in Laemmli's buffer [Laemmli, 1970] and analyzed

by 2D-SDS-PAGE and subjected to fluorography as described previously [Buhi et al., 1995]. All X-ray films were exposed for 14 days at -80°C and developed.

Protein Fractionation

Explant culture media conditioned by isthmus tissue on Days 0, 2, and 12 of early pregnancy, were pooled and subjected to gel filtration chromatography on a Sepharose CL-6B column as described previously [Buhi et al., 1990] with some modifications. The Sepharose CL-6B column (1.8 x 92 cm) was equilibrated in column buffer [10 mM Tris-HCl, 0.4 M NaCl, 0.02% (w/v) NaN_3 ; pH 7.5] at 4°C . Culture media (7 ml) centrifuged at $2,200 \times g$ for 10 minutes at 4°C to remove particulate material, was added slowly to the column. After collection of the void volume, elution profiles were generated by collecting 2 ml fractions, measuring protein (absorption at 280 nm) and determining radioactivity in each fraction. Chromatographic peaks corresponding to the elution of ovalbumin (45,000 M_r standard) were pooled from individual column runs. Immunoglobulins were removed from pooled fractions by incubation with Protein A-Sepharose beads in 10 mM phosphate-buffered saline (PBS), pH 8.0, overnight at 4°C . Beads were then separated from the supernatant by centrifugation at $1,700 \times g$ for 10 minutes at 4°C , washed 3x in PBS, and pooled supernatants dialyzed against dH_2O (2 changes, 4 L each, 24 h each, 4°C). The dialyzed sample was analyzed for protein content and radioactivity, as indicated above, and lyophilized. Lyophilized samples were then resuspended in column buffer (1ml/15 mg protein) and further fractionated on a Sephadex G-100 column (1.5 x 75 cm) at 4°C . The Sephadex G-100 column was calibrated previously with Blue Dextran, apotransferrin, ovalbumin and cytochrome C as molecular weight markers. Elution profiles were generated using methodology

established for the Sepharose CL-6B column. The pooled chromatographic peaks from individual column runs corresponding to the 45,000 M_r standard were utilized for subsequent Western blotting and N-terminal amino acid sequence analysis. Purification at each step was examined by 2D-SDS-PAGE and fluorography [Buhi et al., 1990].

Western Blotting and Sequence Analysis

The pooled chromatographic peaks containing the 45,000 M_r protein were separated by Tris-tricine 2D-SDS-PAGE [Schagger and von Jagow, 1987]. Proteins were then transferred by semi-dry electrophoresis (Milli-Blot SDE system, Millipore Corp, Bedford, CA) to a PVDF membrane as described previously [Buhi et al., 1995] with some modifications. Following separation of proteins by Tris-tricine 2D-SDS-PAGE, the gel was rinsed in three changes of dH_2O for 5 min each, then equilibrated in 25 mM Tris-HCl buffer (pH 9.4) containing 2% (w/v) SDS, for 30 min. The gel was washed in three changes of dH_2O for 5 min, and incubated in two changes of cathode C buffer (25 mM Tris-HCl, 5.25 g/L norleucine, 10% (v/v) methanol; pH 9.4) for 10 min at room temperature. After assembly of the TransUnit sandwich, proteins were transferred for 1 h under constant current (2.5 mA per cm^2). Subsequently, the membrane was washed (dH_2O , 5 min), and stained in 0.1% (w/v) Coomassie blue R-250 in 50% (v/v) methanol for 1 min. The membrane was destained (50% methanol, 5 min) with constant rocking and rinsed in three changes of dH_2O . The PVDF membrane was allowed to air dry, proteins corresponding to the 45,000 M_r protein were excised, and subjected to N-terminal amino acid microsequencing at the Interdisciplinary Center for Biotechnology Research (ICBR) facility using a 470A gas phase protein sequencer (Applied Biosystems) with an on-line analytical HPLC system. The peptide sequence was

analyzed with the National Center for Biotechnology Information (NCBI) BLAST program [Altschul et al., 1990].

After identification of the 45,000 M_r protein as porcine PAI-1 (see Results), a one-step partial-purification method was employed using heparin-agarose affinity column chromatography. PAI-1 has been shown previously to quantitatively bind to heparin-Sepharose and elute with increasing concentrations of NaCl [Ehrlich et al., 1991]. Isthmic-conditioned culture media (Day 12 pregnant and Day 1 cyclic) were pooled, centrifuged as described above, diluted (1:3) in 20 mM Tris-HCl (pH 7.6, 4° C) containing 0.02% (w/v) NaN₃, and slowly loaded onto a heparin-agarose column (2.5 x 8.2 cm) at 4° C. PAI-1 was eluted utilizing stepwise increments of NaCl (0.1-3.0 M), and the protein pooled and dialyzed against dH₂O (two changes, 24 h each, 4L each, 4° C). Protein content was determined as above, and aliquots containing 0.5 mg of protein lyophilized and used for immunoprecipitation. Fractions were analyzed for the presence of PAI-1 by 2D-SDS-PAGE and fluorographic analysis.

Immunoprecipitation

PAI-1, semi-purified by heparin-agarose affinity column chromatography, was immunoprecipitated with a polyclonal rabbit anti-hPAI-1 antiserum (kindly provided by Dr. Schleef, Scripps Institute, La Jolla, CA). Lyophilized protein samples (0.5 mg) were solubilized in 900 µl of NET buffer [50 mM Tris-HCl, 0.15 M NaCl, 0.1% (v/v) Nonidet P-40, 1 mM EDTA, 0.25% (w/v) gelatin, and 0.02% (w/v) NaN₃; pH 7.5 at 25° C]. Protein A-Sepharose beads were equilibrated in NET buffer and 100 µl of swollen beads were incubated with 100 µl of either undiluted PAI-1 antiserum or NRS and 300 µl of NET buffer for 1 h at 25° C. After incubation, Protein A-Sepharose beads were pulse-

centrifuged (Beckman, microcentrifuge) for 30 sec and washed in three changes of NET buffer (0.5 ml). Semi-purified PAI-1 protein (100-200 μ l) was then incubated with NRS or rabbit anti-hPAI-1 antibody-coated beads (200 μ l), respectively, for 2 h at 25^o C with constant rotation. Complexes were pelleted by centrifugation and washed as above. Proteins conjugated to the Protein A-Sepharose beads were solubilized in Laemmli buffer [Laemmli, 1970], boiled for 3 min, separated on a 10% (w/v) 1D-SDS-PAGE gel and subjected to fluorography [Bui et al., 1995]. Heparin-agarose fractionated proteins, which were not used for immunoprecipitation, were removed from solution using standard acetone precipitation procedures [Harlow and Lane, 1988] and solubilized in Laemmli buffer as above for positive radiolabeled PAI-1 identification.

Immunocytochemistry

A polyclonal affinity-purified goat anti-hPAI-1 was used to immunolocalize PAI-1 in porcine oviductal tissues from cyclic and early pregnant animals. To compare distribution of PAI-1 in cyclic and early pregnant animals, infundibulum, ampulla and isthmic tissues were collected on Days 0, 2, and 12, (n=3 animals/day) and immunocytochemistry performed as described [Chagini et al., 1992]. Tissues were cut into 5 mm portions, fixed in Bouin's solution, embedded in paraffin, sectioned (0.5 μ m), and mounted on precoated glass slides. A goat Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA) was used according to the manufacturer's instructions. Controls included use of an affinity-purified goat IgG and the absence of primary antibody. Goat anti-hPAI-1 and goat IgG were used at a dilution of 1:10 in PBS (pH 7.4).

Immunogold Electron Microscopy (EM)

Oviductal tissue from the three segments of Day 0 non-pregnant and Day 9 pregnant crossbred gilts, were fixed for 1 h in PBS, pH 7.4, containing 0.5% (v/v) glutaraldehyde, 4% (v/v) paraformaldehyde at 4⁰ C. The two days selected were times of elevated estrogen (Day 0) or progesterone (Day 9) production. After fixation and rinsing in PBS, tissues were dehydrated in graded ethanol series and embedded in Unicryl (British BioCell International, UK) under UV light at -10⁰ C for 2 days. Thin sections (0.5 mm) were cut and collected on Formvar-coated 100 mesh nickel grids, and PAI-1 antigen detected by immunogold labeling. The polyclonal rabbit anti-human PAI-1 and preimmune rabbit sera, diluted 1:1000 in a high salt Tween buffer (0.02 M Tris-HCl, 0.5 M NaCl, 1% [v/v] Tween 20, pH 7.2) supplemented with 1% (w/v) ovalbumin, were incubated overnight with grids in a humid chamber at 4⁰ C. Sections were then incubated with a secondary antibody (goat anti-rabbit IgG, 1:30 dilution in PBS) conjugated to 18 nm colloidal gold (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA) for 1 h at room temperature. Sections were then post-stained with 2% (w/v) uranyl acetate and Reynolds lead citrate. Grids were examined on a Hitachi H-7000 transmission electron microscope (Hitachi Scientific Instruments, Danbury, CT). Digital micrographs were taken on a Gatan BioScan/Digital Micrograph 2.5 (Gatan Inc, Pleasanton, CA).

Results

Electrophoretic Analysis

Representative 2D-SDS-PAGE and fluorographic analyses of explant culture media from the infundibulum, ampulla, and isthmus containing radiolabeled *de novo*

synthesized proteins are shown in Figure 3-1. The 45,000 Mr protein, found in all three segments, is seen as the major radiolabeled protein from the isthmus portion of the oviduct (Figure 3-1C). The level of protein expression appears to be reduced in the ampulla (Figure 3-1B) and nearly absent in the infundibulum (Figure 3-1A). Thus, the 45,000 Mr protein appears to have a specific spatial synthesis and release in the oviduct. This specific spatial expression is similar to that reported for the matrix metalloproteinase inhibitor, TIMP-1, in the oviduct [Buhi et al., 1996] (Figure 3-1).

Purification, Blotting, and N-terminal Sequencing

With identification of the 45,000 Mr protein as the major radiolabeled protein of the isthmus, the next objective was to purify this protein for N-terminal sequencing. A two-step gel-filtration chromatographic procedure was developed in order to separate and enrich this protein. Elution profiles for protein and radioactivity from Sepharose CL-6B and Sephadex G-100 columns are shown in Figure 3-2A and 3-2B, respectively. Fractionation of explant culture media on these two columns, each showed two peaks of radioactivity. The broad second peak in both graphs, contained the greatest amount of radioactivity and included the 45,000 Mr protein. A representative fluorograph of the 45,000 Mr protein after fractionation on a Sephadex G-100 column is shown in Figure 3-2C. This protein appears to contain at least 5 isoelectric species, of which 3 species, including an acidic and basic species, were submitted for N-terminal amino acid sequence analysis. A search of protein, RNA and DNA data banks indicated that the derived N-terminal amino acid sequence of 26 amino acids, identical for all three isoelectric species, was 96% identical (100% similar) to porcine PAI-1 (Figure 3-3). This sequence (1-26) corresponded to amino acid positions 20-46 of mature PAI-1 protein,

indicating removal of the hydrophobic leader peptide prior to release from the cell [Bijnens et al., 1997].

Immunoprecipitation

To confirm that the 45,000 M_r protein identified by N-terminal amino acid microsequencing was PAI-1, an anti-hPAI-1 serum was used for immunoprecipitation of this protein from isthmus culture media after fractionation by heparin-agarose affinity column chromatography. Fractionated culture media containing *de novo* synthesized radiolabeled proteins were treated with either Protein A-complexed rabbit anti-hPAI-1 serum or Protein A-complexed NRS. Bound proteins were solubilized and examined by 1D-SDS-PAGE and fluorography (Figure 3-4A and 3-4B). As shown in Figure 3-4B, anti-hPAI-1 serum specifically recognized and precipitated radiolabeled PAI-1, while NRS did not. These results indicate that this antibody does not cross-react with other radiolabeled proteins present within the fractionated culture media. Western Immunoblot analysis showed that the anti-hPAI-1 antisera cross-reacted with only 2-3 minor unlabeled proteins, possibly of transudate origin. The primary protein recognized by this antibody on the Western Immunoblot was the PAI-1 family (5-6 isoelectric species) (data not shown).

Immunocytochemistry

With identification of PAI-1, the next objective was to examine its distribution throughout the oviduct on Days 0, 2, and 12 of the estrous cycle or early pregnancy. Immunoreactive PAI-1 was detected in all three segments of the oviduct regardless of day of cycle examined and no differences in staining intensity could be detected between days (infundibulum not shown). Representative data of the immunocytochemical

localization in the isthmus on these days is shown in Figure 3-5. No difference in staining intensity could be detected (subjective visualization of three representative animals) between pregnant and cyclic tissues within the three segments (Figure 3-6). PAI-1 was localized primarily within the oviductal epithelium, while only background staining could be identified within muscle and stroma tissue (Data not shown for muscle and stroma). Here, PAI-1 appeared to be heavily concentrated at the apical region of the epithelium (Figure 3-5C, arrow) with little staining found in the basal region. However, PAI-1 immunoreactivity was also associated within cells lining blood vessels in the stroma. Immunocytochemistry pictures shown are representative of 3 animals/day. Because staining intensities varied between each animal examined, subjective comparisons on the level of staining intensity between pregnant and cyclic gilts were not made.

Electron Microscopy

Since immunocytochemistry was not able to resolve PAI-1 association with specific cellular structures or organelles within the epithelium, an examination of PAI-1 distribution within the epithelium was performed using electron microscopic immunogold localization. PAI-1 was found primarily in epithelium of isthmic non-ciliated secretory cells and appeared to be concentrated in putative secretory granules at or near the apical border of cells in both Day 0 non-pregnant and Day 9 pregnant animals (Figures 3-7A and 3-8A). In addition, this protein was also found to be associated with the isthmic luminal epithelial border and cilia (Figures 3-7B and 3-8B). Localization of PAI-1 within ampullary epithelium (both ciliated and non-ciliated) was negligible (Figure 3-8C). Isthmic tissue incubated with preimmune sera (control) showed no labeling

within secretory granules or at the epithelial border (Figure 3-8D). As detailed in the immunoprecipitation results, the antibody employed showed no potential cross-reactions with other proteins found within oviduct-conditioned culture medium.

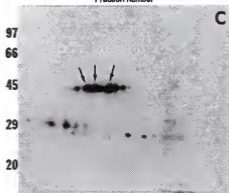
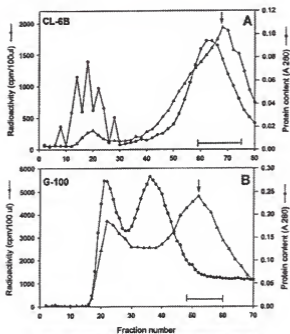
Figure 3-1. Representative fluorograph after 2D-SDS-PAGE separation of [^3H]-labeled proteins (100,000 cpm) from explant culture media conditioned by the three segments of the oviduct.

Tissue was collected from the A) infundibulum, B) ampulla or C) isthmus of Large White X Landrace gilts on Day 2 of pregnancy. The 45,000 M_r protein is indicated by an arrow in each panel. TIMP-1 is marked by an arrowhead only in panel C. Molecular weight markers ($\times 10^3$) are indicated and the pH gradient runs from left (pH 8) to right (pH 4).



Figure 3-2. Representative Sepharose CL-6B and Sephadex G-100 fractionations of [^3H]-labeled proteins in isthmus-conditioned medium.

Fractions (2 ml) collected from the CL-6B column elution (Panel A) were counted for radioactivity and measured for protein (see Methods). In Panel A, tubes 58-75 (peak 2, bracket) containing the 45,000 M_r protein, were pooled, treated with Protein A beads to remove immunoglobulins and further fractionated on a G-100 column (Panel B). In panel B, tubes 48-60 (peak 2, bracket), containing the 45,000 M_r protein were pooled, and separated by Tris-tricine 2D-SDS-PAGE for electroblotting. Arrows in panel A and B reflect elution point of 45,000 M_r ovalbumin molecular weight standard. Panel C, shows a representative fluorograph of the 45,000 M_r protein after chromatographic separation by Tris-tricine 2D-SDS-PAGE. Three isoelectric species (marked with arrows) were excised and subjected to N-terminal amino acid sequence analysis. Molecular weight markers ($\times 10^3$) are indicated and the pH gradient runs from left (pH 8) to right (pH 4).



20	25	30	35	40	45	Porcine PAI-1
EGSAS	SHHQS	LAARL	ATDFG	VKVFR	QV	
EGSAS	SHHQS	LAARL	ATDFG	VKXFR	QV	45,000 Mr isthmus protein
1	5	10	15	20	25	

Figure 3-3. Comparison of the identified N-terminal amino acid sequence of the 45,000 Mr protein from isthmus-conditioned culture media to the N-terminal sequence of porcine PAI-1.

Proteins were fractionated by size-exclusion chromatography using a Sepharose CL-6B and Sephadex G-100 column, separated by Tris-tricine 2D-SDS-PAGE, transferred by electroblotting to PVDF membranes and subjected to N-terminal amino acid microsequence analysis. X (residue 22) indicates an undetermined amino acid residue. A 96% sequence identity was found to porcine plasminogen activator inhibitor-1 (Accession # 1870170).

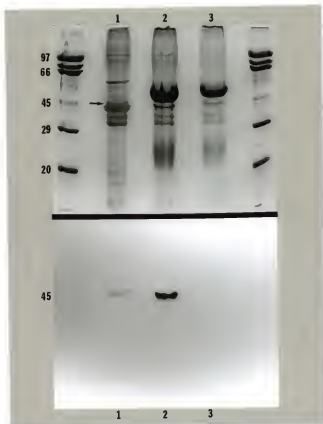


Figure 3-4. Immunoprecipitation of plasminogen activator inhibitor-1 from porcine isthmus-conditioned culture media after fractionation on a heparin-agarose affinity column and elution with 0.4 M NaCl.

A representative one-dimensional SDS-polyacrylamide gel (Panel A) containing Coomassie blue-stained proteins from either a non-precipitated sample (lane 1), proteins precipitated using a rabbit anti-human PAI-1 (lane 2) or normal rabbit sera (lane 3). Although not visible on the gel, the arrow indicates the apparent location of PAI-1 protein. Panel B shows a fluorograph generated from the same gel in Panel A. Lanes are as indicated above. PAI-1 found in semi-purified culture media, was specifically precipitated by antibody (lane 2), but not normal rabbit serum (lane 3).

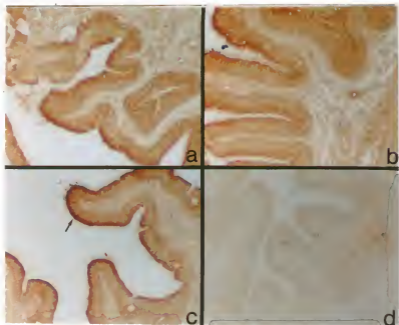


Figure 3-5. Representative immunocytochemical localization of PAI-1 in the isthmus from crossbred gilts on Day 0 (Panel A), Day 2 (Panel B) or Day 12 (Panel C) of pregnancy.

The control is shown in Panel D. Arrow indicates intense staining of PAI-1 within the apical region of isthmic epithelium. X 20.

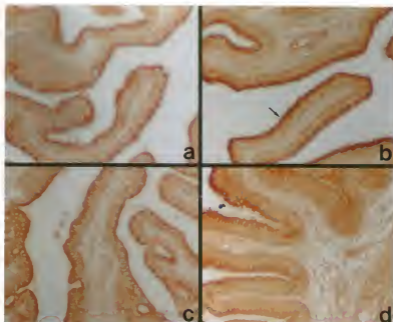


Figure 3-6. Representative immunocytochemical localization of PAI-1 in oviductal tissue from Day 2 cyclic (Panel A and C) and Day 2 pregnant (Panel B and D) crossbred gilts from the isthmus (Panel C and D) and ampulla (Panel A and B).

The infundibulum is not shown. Arrow indicates intense staining of PAI-1 within the apical region of the epithelium. X 20.

Figure 3-7. Immunogold labeling of PAI-1 within oviductal tissue from a Day 0 non-pregnant crossbred gilt.

Gold particles are seen in secretory granules of isthmic non-ciliated cells (Panel A, arrow) as well as associated with the epithelial luminal border and cilia (Panel B, arrow). X 20,000.

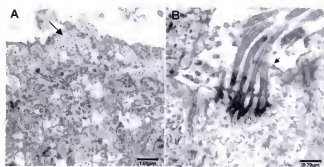
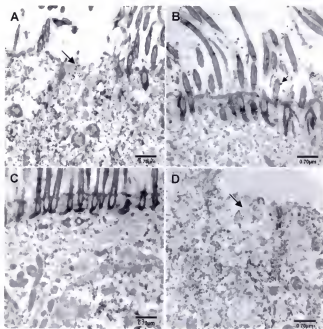


Figure 3-8. Immunogold labeling of PAI-1 within oviductal tissue from a Day 9 pregnant crossbred gilt.

Gold particles can be seen clustered within putative secretory granules of isthmic non-ciliated cells (Panel A, arrow) and associated with the epithelial luminal border and cilia (Panel B, arrow). Gold particles were not detected in secretory granules of the ampulla although some scattered particles were associated with cilia (Panel C). Immunogold staining was not detected in the isthmus treated with preimmune serum (Panel D, arrow). X 20,000.



Discussion

The mammalian oviduct secretes numerous *de novo* synthesized proteins into the oviductal lumen, some of which are present before ovulation, or during fertilization and early cleavage-stage embryonic development. In order to characterize the 14 major *de novo* proteins secreted by the porcine oviduct, explant tissue (infundibulum, ampulla, and isthmus), was incubated with [3 H]-leucine, and synthesized and secreted proteins separated by 2D-SDS-PAGE, detected by fluorography, and characterized by molecular weight and isoelectric points [Buhi et al., 1990]. Two of these proteins have been extensively examined, while others remain unidentified [Buhi et al., 1997], including a 45,000 M_r protein synthesized primarily by the isthmic portion of the oviduct. Sequence analyses revealed that this isthmic protein was porcine plasminogen activator inhibitor-1. Recently, an unidentified 45,000 M_r protein found in bovine oviductal fluid was shown to associate with the zona pellucida of bovine oocytes [Staros and Killian, 1998] and may be the protein under investigation.

PAI-1, a member of the serpin family of serine protease inhibitors, is the primary inhibitor of urokinase plasminogen activator (uPA) and tissue-type plasminogen activator (tPA). It is a glycoprotein consisting of 379 amino acids with an apparent molecular mass of 45,000 [Andreasen et al., 1990]. Among the serpins, PAI-1 is secreted in an active form that rapidly converts to an inactive latent form that can be reactivated by phospholipids and a variety of denaturants [Hekman and Loskutoff, 1985]. The inhibitory activity of PAI-1, however, is stabilized by vitronectin in either serum or extracellular matrix (ECM), thus preventing transformation to the inactive latent form [Declerck et al., 1988]. Both tPA and uPA initiate proteolysis by converting plasminogen

to the broad-specificity enzyme plasmin. This extracellular protease is reported to be involved in the remodeling of ECM, fibrinolysis, cell migration, and tumor metastasis [Andreasen et al., 1990]. Plasmin can also activate pro-matrix metalloproteinases (MMPs) thereby regulating the pericellular activation cascade leading to ECM degradation [Murphy et al., 1992]. Complex control of this activation cascade is regulated by PAI-1 and tissue inhibitors of matrix metalloproteinases (TIMPs).

Immunoprecipitation confirmed the presence of PAI-1 which in the oviduct, appeared to consist of at least five or more isoelectric species as shown by fluorographic analysis, N-terminal amino acid sequencing and immunoblot analysis (data not shown for immunoblot). The presence of these isoforms suggests that newly synthesized and secreted PAI-1 was post-translationally modified. One modification was characterized as glycosylation with incorporation of [^3H]-glucosamine into PAI-1 during tissue explant culture [Buhi et al., 1990]. Localization and synthesis of PAI-1 appears to mimic a matrix metalloproteinase inhibitor from the oviduct, TIMP-1 [Buhi et al., 1996]. Both TIMP-1 and PAI-1 show a similar spatial expression by the oviduct segments, with a greater expression in the isthmus relative to either the ampulla or infundibulum. This would suggest an important function relative to its spatial expression. The isthmus portion of the oviduct has been described as a spermatozoa reservoir where sperm undergo capacitation and hyperactivation [Suarez, 1998]. In the pig, the ampulla-isthmic junction is the location of fertilization and early cleavage-stage embryonic development [Buhi et al., 1997]. This protein may therefore facilitate or regulate these important reproductive events that occur within or near the isthmus. However, PAI-1 may act in other segments of the oviduct as well, due to retrograde movement of oviductal fluid into

the peritoneal cavity during estrus [Hunter, 1988]. Localization of PAI-1 in the infundibulum and ampulla epithelium and protein secretion by explant tissues, suggests that these segments may also contribute to luminal PAI-1, although synthesis of PAI-1 in these segments appears to be very low.

Fibrin deposits have been located on the tubal mucosa of the oviduct, which could possibly interact with the ECM components of the zona pellucida and prevent tubal transport [Liedholm and Astedt, 1975]. Liedholm and Astedt (1975), observed fibrinolytic activity associated with the unfertilized ovum in rats and suggested that this activity may be involved with the prevention of adhesion to fibrin deposits that may hinder gamete transport. This activity was also shown to be associated with spermatozoa. Prevention of cellular adhesion to the oviductal mucosa and fibrin deposits might also be regulated by uPA and tPA. The developing embryo may produce uPA/tPA in response to signals from the surrounding oviductal environment. Fibrin has been shown to increase expression of uPA mRNA and protein in 3.5 day old uterine embryos of the mouse [Zhang et al., 1996]. Both mouse and rat preimplantation embryos have been shown to have tPA activity [Zhang et al., 1992, Carroll et al., 1993] and uPA activity [Zhang et al., 1994, Harvey et al., 1995]. Expression of this proteolytic activity, or the respective mRNA, was found to be developmental- and stage-specific. While the fibrinolytic activity of the ovum and proteinase expression by the embryo may facilitate their transport through the oviduct, these molecules are potent modulators of their immediate environment with respect to ECM remodeling. PAI-1, an important regulator of both fibrinolysis and plasminogen activators, may act as a stabilizing or counter-regulatory factor for maintaining ECM integrity of the oviductal epithelium. Therefore, production

of PAI-1 by the oviduct might act to prevent premature nidation of the preimplantation embryo. While the pig has noninvasive placentation, the trophoblast of the pig has invasive potential as shown by its transfer to ectopic sites [Samuel, 1971]. Part of this invasive potential may be due to fibrinolytic and plasminogen activator activity of the embryo. Liedholm and Astedt (1975) suggested that the fibrinolytic activity of the ovum may be depressed by an inhibitor of plasminogen activation. Plasmin-induced proteolysis has been shown to be important for implantation in invasive species such as the mouse [Sappino et al., 1989, Zhang et al., 1996]. However, in the pig, an endometrial inhibitor of plasmin production has been suggested to protect the endometrium from the blastocyst-induced proteolysis during placentation [Fazleabas et al., 1982]. PAI-1 secretion within the oviduct, may therefore have a similar function to that of the endometrial plasmin inhibitor.

Immunolocalization results indicated that PAI-1 is localized within the oviductal epithelium and is heavily concentrated near the apical membrane, suggesting secretion into the lumen. Immunogold EM of the isthmus, revealed that PAI-1 was located in putative secretory granules within the lumen and associated with cilia. While PAI-1 was localized to the ampulla using immunocytochemistry, immunogold electron microscopy was unable to detect its presence. This may reflect a difference in specificity and dilutions of two different antibodies used, difference in tissue sections examined between the two different procedures and 2D-SDS-PAGE and fluorographic analyses, which suggest that PAI-1 protein secretion is low in the ampulla. Both immunocytochemistry and fluorographic analysis have shown that PAI-1 is localized in the ampulla and infundibulum, however, the inability to locate immunoreactivity utilizing electron

microscopy may be that protein synthesis of this molecule is very low in these segments and is present in secretory granules in very small amounts. PAI-1 was found in secretory granules from isthmic tissue exposed to either a high estrogen (Day 0 cyclic) or high progesterone (Day 9 pregnant) environment indicating that this protein is synthesized throughout the estrous cycle or early pregnancy. Evidence from our laboratory (unpublished) indicates that PAI-1 secretion may vary during the estrous cycle or early pregnancy and its synthesis might be controlled by ovarian steroids. Reports on PAI-1 regulation in endometrial stromal and decidual cell cultures suggest that this protein is up-regulated by progesterone, while estrogen antagonizes this effect [Schatz and Lockwood, 1993]. The oviduct along with the uterus, is a major target for ovarian steroids and steroid-modulated and cycle-specific changes have been noted for two other *de novo* synthesized products of the epithelium, pOSP and TIMP-1 [Buhi et al., 1997].

Because the zona pellucida can be subject to proteolytic degradation, oviductal PAI-1 may also protect the integrity of the zona pellucida from embryonic or oviductal plasminogen activator activity. To our knowledge, PAI-1 mRNA or activity has not been examined in the early cleavage-stage embryo or in the oviduct. The porcine zona pellucida of oviductal oocytes or embryos was found to be more resistant to proteolytic degradation than that of either follicular oocytes or embryos collected from the uterine environment [Broermann et al., 1989], suggesting a potential interaction of oviductal protease inhibitors with the oocyte/embryo. Changes in resistance of the zona pellucida to proteases may be dependent upon addition of glycoproteins/inhibitors obtained during transit through the oviduct. Thus, an oviduct-specific factor may act to protect the zona pellucida and embryo from degradation by proteolytic enzymes. Proteases are present in

oviductal flush and include the plasminogen activators and matrix metalloproteinases (Kouba AJ, unpublished). PAI-1 working together with TIMP-1, may act to tightly regulate this proteolytic activity. Plasminogen, the natural substrate for uPA and tPA, is found in many extracellular fluids including seminal plasma [Kobayashi et al., 1992] and follicular fluid [Beers, 1975], and may be enriched in oviductal fluid at or near the time of fertilization. Estrogens have been shown to stimulate the uptake of plasminogen from plasma by the mouse uterus [Finlay et al., 1983] and a similar function may occur in the oviduct during estrus. Plasminogen has been shown to bind to mouse spermatozoa, oocytes, and cumulus cells, which enhanced the local generation of plasmin [Huarte et al., 1993] and this binding allows for PA-induced proteolysis to discrete focal areas. Huarte et al. (1993) also showed that addition of plasminogen or antibodies to plasmin during *in vitro* fertilization could increase and decrease, respectively, the fertilization rate. Therefore, PAI-1 within the oviduct may inhibit oocyte or embryonic generation of plasmin from plasminogen, due to their inherent tPA and uPA activity, thus maintaining integrity of the zona pellucida while not affecting fertilization.

Further objectives will be to evaluate the biological role of PAI-1 within the oviduct. PAI-1 may act in a autocrine/paracrine fashion to prevent proteolytic degradation of ovulated oocytes or early embryos in the oviduct or uterus, prevent premature hatching, regulate ECM remodeling of the oviduct or early embryo, inhibit embryonic invasion of the oviductal lining, and promote embryonic development.

CHAPTER 4

OIDUCTAL PLASMINOGEN ACTIVATOR INHIBITOR-1 (PAI-1) PROTEIN, mRNA, AND HORMONAL REGULATION

Introduction

The mammalian oviduct is host to both male and female gametes as well as the early cleavage-stage embryo. Oviductal fluid provides an important medium that facilitates gamete union/fertilization and nurtures the resultant zygote and developing embryo. Oviduct fluid is also a potential source of signal molecules such as growth factors, protease inhibitors, and oviduct-specific secretory glycoproteins [Buhi et al., 1997]. Several proteins have been shown to be synthesized *de novo* and secreted into culture medium from oviductal explant cultures [Buhi et al., 1990]. Two of these proteins have been identified as TIMP-1 [Buhi et al., 1996] and PAI-1 [refer to Chapter 3]. These protease inhibitors have been described as tightly regulating an activation cascade of proteases which leads to degradation and/or remodeling of the extracellular matrix (ECM). Since gametes or early cleavage-stage embryos are in intimate contact with the oviductal epithelium, a means is provided for cell-cell interaction within their immediate environment [Hunter, 1988]. Buhi et al. (1996) and Kouba et al. (Chapter 3) have proposed that the protease inhibitors, PAI-1 and TIMP-1, provide protection against enzymatic degradation and prevent adhesion and invasion of the early cleavage-stage embryos within the porcine oviduct.

Experiments in mice have shown that the blastocyst can easily implant in a wide variety of sites within the body upon transplantation [Kirby, 1963], yet the oviduct in non-primates is extraordinarily resistant to tubal implantation [Bronson and McLaren, 1970]. The early cleavage-stage embryo has been shown to produce both urokinase plasminogen activator (uPA) [Zhang et al., 1994, Harvey et al., 1995] and tissue-type plasminogen activator (tPA) [Carroll et al., 1993, Zhang et al., 1992], both of which can lead to the generation of plasmin, a broad specificity enzyme that can initiate remodeling and degradation of the ECM. Although the embryo is in an environment containing proteases such as uPA and tPA, as well as their substrate plasminogen [Huarte et al., 1993], ovulated oviductal oocytes are extremely resistant to proteolytic degradation [Broermann et al., 1989], and oviducts are strongly resistant to adhesion and implantation [Jansen, 1984]. PAI-1 and TIMP-1 have been suggested to play a role in these phenomenon [Buhi et al., 1996, Kouba et al., Chapter 3]. The inhibitors PAI-1 and TIMP-1, are major *de novo* synthesized and secreted proteins of the isthmic portion of the oviduct, the location of early cleavage-stage embryonic development [Buhi et al., 1996, Kouba et al., Chapter 3]. Further, PAI-1 protein was found localized within secretory granules of the isthmic epithelium and associated with isthmic cilia [refer to Chapter 3].

Another oviductal protein which has been characterized is the porcine oviduct-specific glycoprotein (pOSP), an estrogen-regulated protein synthesized primarily by the ampulla and infundibulum [Buhi et al., 1996]. Immunogold electron microscopy labeling showed that pOSP associates with the zona pellucida, perivitelline space and vitelline or blastomere membranes of ovulated oocytes and early embryos, respectively [Buhi et al.,

1993]. Protein, mRNA, and hormonal regulation of TIMP-1 and pOSP in the oviduct has been evaluated throughout the estrous cycle and early pregnancy [Bui et al., 1996]. Elucidation of spatial and temporal secretion of PAI-1 is significant to our understanding of how the cleavage-stage embryo may be protected in a protease rich environment and provide resistance to implantation.

The specific objectives of this study were: 1) to examine *de novo* synthesis and secretion of radiolabeled PAI-1 during early pregnancy in two breeds of pigs, the standard European Large White and the highly prolific Chinese Meishan, 2) to determine PAI-1 levels in oviduct flushes from early pregnant crossbred gilts, 3) to evaluate levels of PAI-1 mRNA in crossbred gilts during the estrous cycle, early pregnancy, and after ovariectomy (OVX) and steroid-hormone replacement therapy and 4) to determine specific spatial differences in expression of PAI-1 mRNA and protein between different segments of the oviduct.

Materials and Methods

Materials

Acrylamide, N,N' diallyltartardiamide, urea, Nonidet P-40, and Sodium dodecyl sulfate were from Gallard-Schlesinger (Carle Place, NY); X-Omat AR film and photography reagents were a product of Eastman Kodak Co. (Rochester, NY); leucine and protein standards were acquired from Sigma-Aldrich (St. Louis, MO); ampholines were from Pharmacia-Biotech (Piscataway, NJ); all other supplies and reagents for gel electrophoresis were procured from either Bio-Rad Laboratories (Richmond, CA) or Fisher Scientific (Orlando, FL); and all medium and culture supplies were purchased from Life Technologies (Grand Island, NY). L-[4,5-³H]leucine (specific activity, 120

Ci/mmol) was obtained from Amersham (Arlington Heights, IL). RNA hybridization was performed using the enhanced chemiluminescent kit (ECLTM) purchased from Amersham Pharmacia-Biotech (Piscataway, NJ)

Tissue Collection

Estrous cycle Sexually mature crossbred gilts (Yorkshire X Duroc X Hampshire) were observed daily for behavioral estrus for at least two estrous cycles in the presence of an intact boar. The first day of standing estrus was designated Day 0 and animals were taken to the abattoir for slaughter on Days 0, 1, 2, 4, 8, 10, 12, 15, and 18 of the estrous cycle (n=3/day). After exsanguination, reproductive tracts were collected aseptically and the oviduct separated by gross dissection into the infundibulum, ampulla, and isthmus. Tissues from one oviduct per animal were frozen immediately in liquid nitrogen and stored at -80⁰ C for subsequent RNA extraction. The second oviduct was utilized for explant tissue culture as described below.

Ovariectomy Crossbred gilts to be treated with various steroids (n=12) were bilaterally ovariectomized (OVX) on the fourth day following a natural estrus and randomly assigned to one of four treatment groups. Gilts were injected (i.m.) daily for 11 consecutive days (Bui et al., 1992) and treated with the following steroid regimens. Treatment 1 received vehicle (2 ml), corn oil and ethanol (9:1, v/v; n=3); treatment 2 received 100 µg estradiol valerate (EV; n=3); treatment 3 received 200 mg progesterone (P₄) (n=3); treatment 4 received 200 mg P₄ + 100 µg EV. OVX gilts were anesthetized 24 h after the last treatment, and subjected to surgery as described below. The oviduct was flushed as described below, oviductal segments cultured, and/or tissue snap-frozen for RNA analysis.

Pregnancy In order to evaluate PAI-1 message during early pregnancy, crossbred gilts were mated with boars at the onset of estrus and again 24 h later to ensure fertile mating (except animals assigned to Day 0 of pregnancy). Tissues were obtained by surgery (ovariohysterectomy) as detailed below or gilts were taken to the abattoir for slaughter on Days 0, 2, and 12 of early pregnancy (n=3/day). Tissue was collected aseptically, separated into the functional segments and frozen in liquid nitrogen as detailed above. Samples were stored at -80°C for subsequent RNA extraction. Differences in protein secretion of PAI-1 were compared in two breeds of pigs, European Large White and Chinese Meishan. Gilts were observed for behavioral estrus for four estrous cycles, and on Day 0 of the fifth cycle, gilts were bred with a boar of the respective breed as described above. Animals were taken to slaughter and tissues were collected aseptically on Days 0, 2, and 5 of early pregnancy (n=3/day) and cultured as tissue explants as detailed below.

Oviductal flush Oviducts from crossbred pregnant gilts (Days 0, 1, 2, 5, 8, 10, and 12) were flushed after surgery (as detailed below) or sacrifice at the local abattoir. Reproductive tracts were immediately flushed from the fimbria-infundibulum through the isthmus with 5 ml of (modified) Eagles minimum essential medium (MEM) using a 10 ml disposable syringe and 20-gauge needle. Flushed material was collected into a sterile 15 ml conical tube and stored at -20°C . Animal-use protocols were approved by the University of Florida Institutional Animal Care and Use Committee.

Surgery (ovariectomy and ovariohysterectomy) Anesthesia was induced with a combination of Telzol (Fort Dodge, IA; 2.2 mg/kg) and Xylazine (Fort Dodge; 2.2 mg/kg) administered intramuscularly. Following induction of anesthesia, the anesthetic

plane was maintained via inhalation of halothane (Halocarbon Laboratories, River edge, NJ)-oxygen mixture. Gilts were placed in dorsal recumbancy and the abdomen was prepared for midventral laparotomy. The ovaries and/or uterus were excised by ligation of the ovarian pedicles, followed by the broad ligament and the uterine body, cranial to the cervix. The incision was closed and gilts allowed to recover for 24 h.

Explant Culture, Electrophoresis, Fluorography, and Densitometry

Oviductal functional segments (infundibulum, ampulla, isthmus) from early pregnant (Days 0, 2, and 5) Large White or Meishan gilts and steroid-treated OVX crossbred gilts were cultured as previously described (Buhi et al., 1990). Oviducts from pregnant pigs were opened longitudinally and washed in several volumes of (modified) Eagle's minimum essential medium (MEM). Tissue segments were cut into 1- to 3-mm³ sections and explants (500 mg) were cultured in 15 ml of leucine-deficient MEM containing 100 μ Ci of [³H]-leucine on a rocking platform at 39^o C under an atmosphere of 50% N₂: 47.5% O₂: 2.5% CO₂ (v:v:v). After 24 h of culture, media was aspirated, and frozen at -20^o C until analyzed by 2D-SDS-PAGE and fluorography. Prior to electrophoresis, culture medium was dialyzed against 10 mM Tris-HCl buffer (pH 7.6), containing 0.15 M NaCl and 0.02% (w/v) NaN₃, followed by dialysis against dH₂O (2 changes, 4 L each, 24 h each 4^o C). Total protein content of dialyzed culture media was measured by the Bio-Rad microassay (according to manufacture's instructions) and radioactivity measured by liquid scintillation spectrophotometry. Dialyzed culture media (100,000 cpm) from early pregnant and OVX tissues, were lyophilized, solubilized and proteins separated by 2D-SDS-PAGE and subjected to fluorography as previously described [Buhi et al., 1995]. Ampulla and isthmus fluorographs were exposed for 7 days

at -80°C and developed, while infundibulum fluorographs required a longer exposure (14 days). Densitometry of fluorographs were measured on a AlphaImager 2,000 and MultiImage Light Cabinet (Alpha Innotech Co., San Leandro, CA) according to manufacturer's specifications. Data were adjusted for wet weight of tissue and volume of culture media and are expressed as densitometric units/culture/gram of tissue.

Oviductal PAI-1 During Early Pregnancy

PAI-1 was measured in oviduct flushes collected during early pregnancy (Days 0, 1, 2, 5, 8, 10 and 12) using the Immulyse PAI-1 ELISA kit (Biopool International, Ventura, CA). The antibody combination measures active and latent PAI-1, as well as that complexed as tPA/PAI-1 or uPA/PAI-1. Briefly, oviduct flushes were pooled by day, dialyzed, measured for protein content, and lyophilized as described. Lyophilized samples were resuspended in ELISA buffer and 200 μg of sample from each day were added to individual wells of a 96-well plate. The ELISA was run according to manufacturer's instructions. After termination of the reaction, the absorbance (450 nm) was recorded and PAI-1 expressed as pg of PAI-1 per μg of oviductal protein. All samples were run in duplicate and PAI-1 concentrations were determined from the standard curve supplied with the ELISA kit (range 0-50 ng/ml; detection limit 0.9 ng/ml).

RNA Isolation and Analysis

Total cellular RNA was isolated from snap-frozen (-80°C) oviductal tissue (cyclic, pregnant, and OVX) with TRIzol (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. After isolation, total RNA was resuspended in water, and purity and concentration determined by spectrophotometric analysis. For dot blot analyses, 5 μg of total RNA was blotted onto nylon membranes using a vacuum

manifold apparatus (Schleicher and Schuell, Keene, NH) and UV cross-linked (1 minute at 1200 mJ/cm²) to the membrane. A porcine PAI-1 cDNA insert in the pCR 2.1 cloning vector (Invitrogen, Carlsbad, CA) was obtained courtesy of Dr. Paul Declerck, Leuven, Belgium (Bijnens, et al., 1997). Plasmid DNA was digested with *NcoI* restriction enzyme to generate a cDNA probe of 1143 base pairs in length. The insert was gel-purified using the QIA Quick Gel Extraction Kit (Santa Clarita, CA) according to manufacturer's specifications. Sequence of the PAI-1 probe was confirmed using the ABI 373 Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Foster City, CA). The gel-purified porcine PAI-1 cDNA insert was labelled and hybridized using the ECLTM direct nucleic acid labelling and detection system according to manufacturer's specifications. Blots were hybridized overnight in a hybridization incubator (Robbins Scientific, Model 1000, Sunny Vale, CA) at 42^o C, exposed to X-ray film (Fujifilm, Stamford, CT) for 2 h at 4^o C and developed. Hybridization signals were quantified by densitometry using Sigma Gel Scan (Jantel Corporation, San Rafael, CA) and mean background value subtracted. All membranes were reprobed following the chemiluminescent reaction and inactivation of the enzyme label, using a random-primed cDNA corresponding to the coding region of porcine 18S rRNA (courtesy of Dr. Frank Simmen, University of Florida) to confirm equal loading of RNA and mean background value subtracted. The positive control included total RNA (5 µg) taken from the ovarian corpus luteum [Smith et al., 1997], while the negative control was 5 µg of yeast total RNA. The porcine PAI-1 cDNA was characterized previously by Dr. Paul Declerck and was shown to specifically recognize only PAI-1. Northern analysis of the RNA was not done for PAI-1.

Statistical Analyses

All densitometric values obtained from X-ray image analyses were subjected to ANOVA using the General Linear Models procedure of the Statistical Analysis System (SAS Institute Inc., Cary NC, 1988). Values with a $p < 0.1$ were considered significant. The model for PAI-1 protein levels in oviductal segments of early pregnant gilts included main effects of day, breed, segment and all higher level interactions. Data are expressed as least-squares means \pm SEM. PAI-1 protein secretion in OVX steroid-hormone replacement animals was evaluated using a 2×2 factorial design. Densitometric values were subjected to transformation due to heterogeneity of variability and are expressed as the mean \pm SEM. The relationship between PAI-1 levels in oviduct flushes (expressed as $\text{pg}/\mu\text{g}$ oviduct protein) and days of early pregnancy were evaluated by regression analysis.

The model for PAI-1 mRNA in the oviduct during the estrous cycle or early pregnancy included the main effects of day, segment, and day by segment where appropriate. A similar 2×2 factorial design was used to analyze PAI-1 mRNA in OVX steroid-hormone replacement animals. RNA data are presented as least-squares means \pm SEM and have been adjusted for differences in RNA loading by a covariate analysis with 18S rRNA hybridization values. A set of pre-planned orthogonal contrasts were used to evaluate differences for PAI-1 mRNA levels among days of the estrous cycle or early pregnancy. Contrasts shown in Figure 4-4 include comparing Day 2 + Day 12 against other days of the cycle in the isthmus (Panel A) and Day 1 + Day 2 against other days of the cycle in the whole oviduct (Panel B). Contrasts shown in Figure 4-5 included

comparing Day 2 vs. Day 12 and Day 2 + Day 12 vs. Day 0 in each segment (infundibulum, ampulla, and isthmus) for both cyclic and pregnant gilts.

Results

De Novo Synthesis and Secretion of PAI-1 During Early Pregnancy

To evaluate PAI-1 synthesis during early pregnancy, oviductal tissue from the three functional segments of Large White and Meishan pigs, were placed into explant culture and media analyzed by 2D-SDS-PAGE and fluorography. Densitometric analysis of fluorographs revealed changes in PAI-1 secretion patterns during early pregnancy. A greater secretion of PAI-1 ($p<0.01$) was found in the isthmus than in the ampulla portion of the oviduct regardless of day of pregnancy or breed (Figure 4-1). An effect of segment ($p<0.01$) and day ($p<0.1$) were detected for PAI-1 protein, however no differences were detected for breed. Interactions for breed by segment ($p<0.1$), breed by day ($p<0.01$) and breed by segment by day ($p<0.1$) were detected. The Large White had a greater expression of PAI-1 protein in the isthmus on Day 2 of early pregnancy compared to other days examined, while the Meishan had greater amounts on Day 0. Since infundibulum PAI-1 protein was undetectable in fluorographs exposed for 7 days at -80° C, exposure for 14 days was required. The ampulla and isthmic fluorographs could not be measured in a direct comparison with the infundibulum since 14 days resulted in an over exposure of fluorographs from these segments. However, no differences were detected for infundibulum PAI-1 levels between breeds and days of early pregnancy (data not shown).

PAI-1 in Oviduct Flushes During Early Pregnancy

PAI-1 was undetectable by ELISA in individual samples. Therefore, flushings from animals, collected and pooled according to day of early pregnancy, were concentrated and subjected to analysis for PAI-1 by ELISA. Hence, there was only one observation per day. Variation in PAI-1 concentration in concentrated flushes due to stage of early pregnancy was best described by a fifth order regression equation ($R^2=0.55$). As shown in Figure 4-2, PAI-1 in oviductal flushes may peak on Day 2 of early pregnancy. Concentrations were low on Days 1, 5, 10, and 12 and were undetectable on Day 0.

Hormonal Control of PAI-1 Synthesis and Secretion

To examine hormonal control of PAI-1 synthesis by the isthmus, tissue from bilaterally OVX steroid-treated crossbred gilts was placed in culture and media analyzed by 2D-SDS-PAGE, fluorography and densitometry. Densitometric analysis of fluorographs revealed that estrogen stimulated synthesis of PAI-1 protein in the presence of progesterone ($p<0.10$) but that an inhibition of PAI-1 synthesis occurred in the absence of progesterone (Figure 4-3).

PAI-1 mRNA Steady-State Levels During the Estrous Cycle

Using dot-blot hybridization, steady state levels of PAI-1 mRNA were evaluated in whole oviductal tissue throughout the estrous cycle (Days 0 to 18) in crossbred gilts. Relative densitometric values are shown in Figure 4-4B. An effect of Day was found ($p<0.05$) on PAI-1 mRNA expression throughout the estrous cycle, with significantly greater levels of mRNA found on Days 1 and 2 than all other Days examined. No difference could be detected in PAI-1 mRNA levels between Days 1 and 2.

To evaluate steady-state levels of PAI-1 mRNA in the isthmus segment (primary site for PAI-1 synthesis), dot-blot hybridizations were evaluated during the estrous cycle as above. An effect of day ($p=0.08$) was found for PAI-1 mRNA expression in the isthmus during the estrous cycle (Figure 4-4A). Unlike PAI-1 mRNA in the whole oviduct, isthmus PAI-1 mRNA was significantly elevated on Days 2 and 12 of the estrous cycle compared to all other days examined. For all dot-blot analysis, the positive control (corpus luteum) had visible labeling, while yeast RNA did not hybridize with the PAI-1 cDNA probe.

PAI-1 mRNA Steady-State Levels in the Three Functional Segments

Levels of PAI-1 mRNA in crossbred gilts were also characterized in the three oviductal segments on Days 0, 2 and 12 of the estrous cycle or early pregnancy. In cyclic animals, an effect of day ($p<0.01$) and segment ($p<0.01$) were found, however, an interaction was not detected (Figure 4-5). No differences were detected between pregnant and cyclic animals. Orthogonal contrasts of least-squares means revealed that in cyclic animals, Day 2 + Day 12 had a greater ($p<0.01$) expression of PAI-1 mRNA than Day 0 in the infundibulum and ampulla. While mRNA expression on Days 2 + Day 12 were numerically greater than Day 0, no significant differences could be detected in the isthmus on the three days examined. No significant differences could be detected between Day 2 and Day 12 within the three oviduct segments of cyclic gilts.

In pregnant animals, an effect of day ($p<0.01$), segment ($p<0.01$) and an interaction of day by segment ($p<0.05$) was detected for PAI-1 mRNA (Figure 4-5). Similar contrasts as those for cyclic animals, revealed that Day 2 + Day 12 had a greater ($p<0.05$) level of PAI-1 mRNA than Day 0 in the infundibulum and isthmus, but not the

ampulla. A difference between Day 2 and Day 12 was detected in the infundibulum ($p<0.05$) and ampulla ($p<0.01$) but not in the isthmus, with a greater level of PAI-1 mRNA on Day 12 than Day 2.

Hormonal Regulation of PAI-1 mRNA

Oviducts from crossbred gilts, bilaterally OVX and treated with various steroid regimens (corn oil, estrogen, progesterone, or estrogen + progesterone), were examined by dot-blot hybridization. Factorial analysis indicated an interaction ($p<0.05$) for estrogen and progesterone. Progesterone alone significantly increased expression of PAI-1 mRNA, however this expression is dependent upon the absence of estrogen (Figure 4-6).

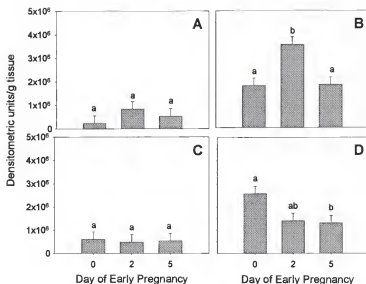


Figure 4-1. Densitometry of PAI-1 protein in fluorographs from 2D-SDS-PAGE analyses of isthmic- and ampulla-conditioned medium (100,000 cpm) from Large White and Meishan gilts during early pregnancy.

Densitometry of [³H]-leucine labeled PAI-1 from; A) Large White, ampulla, B) Large White, isthmus, C) Meishan, ampulla and D) Meishan isthmus on Days 0, 2, and 5 (n=3/day/segment) of early pregnancy. Least-squares means \pm SEM of PAI-1 are expressed as densitometric units/g tissue. Effects of segment ($p < 0.01$), day ($p < 0.1$), breed by segment ($p < 0.1$), breed by day ($p < 0.01$), and breed by segment by day ($p < 0.1$) were detected. Values without common superscripts are significantly different.

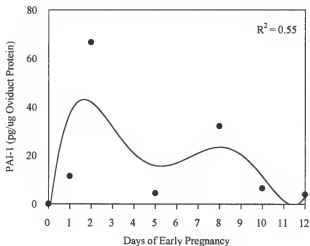


Figure 4-2. PAI-1 protein in oviduct flushes from crossbred gilts during early pregnancy.

Oviductal flushes from early pregnant crossbred gilts were pooled by day (0, 1, 2, 5, 8, 10, 12; $n = 3, 6, 6, 3, 3, 6, 4$ / day, respectively) and measured using an ELISA (see Materials and Methods). Regression analysis is shown as a fifth order polynomial. PAI-1 levels are expressed as pg/ μ g oviduct protein. PAI-1 is shown to peak near Day 2 of early pregnancy.

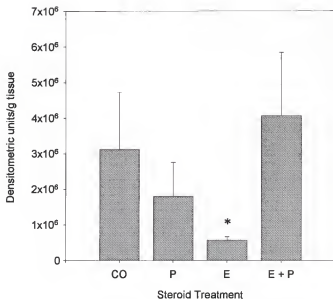


Figure 4-3. Densitometry of PAI-1 protein in fluorographs from 2D-SDS-PAGE analyses of isthmic-conditioned medium (100,000 cpm [³H]-leucine) from OVX crossbred gilts.

Gilts (n=3/treatment) were treated with various steroid regimens (corn oil [CO], estrogen [E], progesterone [P], or estrogen + progesterone [E+P]). Values were transformed and data are expressed as means \pm SEM. Bars with an asterisk are significantly different ($p < 0.05$) from corn oil.

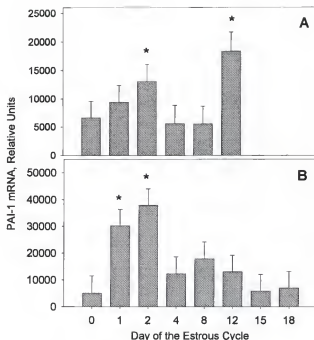


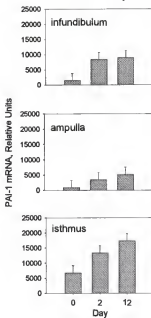
Figure 4-4. Dot-blot hybridization analyses of oviductal total RNA from cyclic crossbred gilts.

Total RNA from the A) isthmus, or B) whole oviduct, were collected from gilts ($n=3/\text{day}$) on Days 0, 1, 2, 4, 8, 12, 15, and 18 of the estrous cycle (isthmus missing Days 15 and 18). Levels of PAI-1 mRNA are shown as least-squares means \pm SEM and were analyzed with the 18S rRNA as a covariate. For whole oviduct, an effect of day was found ($p < 0.05$) and the mean of Days 1 and 2 were significantly greater than all other days examined (marked by asterisk). In the isthmus, an effect of day was found ($p < 0.1$), and Days 2 and 12 were significantly greater than other days examined (marked by asterisk). In order to detect PAI-1 mRNA in whole oviduct (comprised primarily of ampulla tissue) cDNA probe concentrations were increased and hybridization stringency modified compared to results shown for Isthmic PAI-1 mRNA. Therefore, PAI-1 mRNA (relative units) should not be compared between panels A and B.

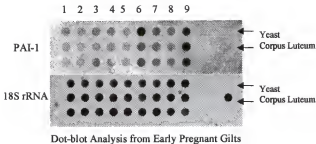
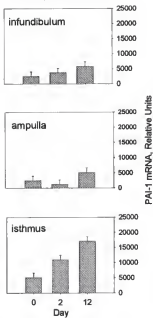
Figure 4-5. Dot-blot hybridization analyses of oviductal total RNA from the infundibulum, ampulla, and isthmus of crossbred gilts.

Hybridization values were compared for gilts (n=3/day/segment) on Days 0, 2, and 12 of the A) estrous cycle or B) early pregnancy for all 3 segments of the oviduct (as shown). Levels of PAI-1 mRNA are shown as least-squares means \pm SEM and were analyzed with the 18S rRNA as a covariate. No differences in mRNA expression were detected between cyclic and pregnant gilts. Refer to results for effects of day, segment and all interactions. The mean of Days 2 and 12 PAI-1 mRNA were found to be significantly greater ($p<0.05$) than Day 0 in the infundibulum and ampulla of cyclic gilts and the infundibulum and isthmus in early pregnant gilts. A representative dot-blot from early pregnant cross-bred gilts is shown at the bottom of Figure 4-5 for both PAI-1 and the 18S rRNA. Rows 1-3 (Day 0), rows 4-6 (Day 2), and rows 7-9 (Day 12); infundibulum (rows 1, 4, and 7), ampulla (rows 2, 5, and 8), and isthmus (rows 3, 6, and 9). A negative control (yeast) and a positive control (corpus luteum) were also included.

A. Estrous Cycle



B. Early Pregnancy



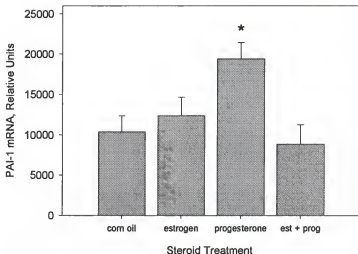


Figure 4-6. Dot-blot hybridization analysis of whole oviductal total RNA from OVX steroid-treated crossbred gilts.

OVX gilts ($n=3/\text{treatment}$) were treated with various steroids (CO, E, P, E+P) as described. Levels of PAI-1 mRNA are shown as least-squares means \pm SEM and were analyzed with the 18S rRNA as a covariate. An interaction was detected for E+P ($p<0.05$). The stimulation of P on PAI-1 mRNA is dependent upon the absence of estrogen. Values with an asterisk are significantly different from corn oil.

Discussion

Several reports have suggested that the oviduct provides an important microenvironment which supports development of early cleavage-stage embryos *in vivo* [Gandolfi, 1995, Boatman, 1997, Buhi et al., 1997]. These researchers suggest that oviductal proteins such as growth factors, cytokines, and the oviduct-specific secretory glycoprotein (OSP) secreted by the oviductal epithelium may sustain or facilitate the processes of fertilization and early embryonic development. In the pig, fourteen *de novo* synthesized and secreted proteins have been described in a limited fashion by relative molecular weight and isoelectric point [Buhi et al., 1990], and steroid-modulated and cycle-specific changes in oviductal secretory proteins have been reported [Buhi et al., 1997]. The beneficial effects of oviductal epithelial cell cocultures on embryonic development to the blastocyst stage is well known [White et al., 1989, Nancarrow and Hill, 1994, Suzuki and Foote, 1995] and the search for embryotrophic molecules secreted by the oviduct is still actively under investigation [Liu et al., 1998]. Recently, PAI-1 was identified in the pig oviduct by western blotting and N-terminal amino acid microsequence analysis [refer to Chapter 3]. This protein was found to be a major *de novo* synthesized and secreted product of the isthmus, was localized to the apical region of oviductal epithelium, and associated with putative secretory granules and cilia of the isthmus [refer to Chapter 3]. With identification of PAI-1 in the pig oviduct, a closer evaluation of its role in this tissue was examined by evaluating expression of its mRNA and protein during the estrous cycle and early pregnancy.

Analysis of PAI-1 mRNA in the bovine oviduct showed a low steady-state expression throughout the estrous cycle [Einspanier et al., 1997]. In contrast, this study clearly demonstrated that PAI-1 transcript and protein synthesis and secretion vary throughout the estrous cycle and early pregnancy. Discrepancies between these studies may be the result of differences between assays, more frequent time periods being evaluated here, species differences, and examination of the three specific segments in our study. Expression of PAI-1 mRNA was identified in all three segments of the oviduct, supporting earlier work which localized PAI-1 protein to the apical region of the infundibulum, ampulla, and isthmic epithelium [Chapter 3]. However, while found in all segments, levels of PAI-1 mRNA were found to be greater in the isthmus than the other two segments indicating the isthmus as the primary site of expression of PAI-1. Day of cycle-dependent expression of PAI-1 mRNA was found in both whole oviduct and isthmic segments. A consistently high level of PAI-1 mRNA on Day 2 of the estrous cycle and/or early pregnancy in both whole oviduct and the isthmus suggest the importance of this protein during fertilization and early embryonic development. Differences observed in PAI-1 mRNA on Day 12, between the isthmus and whole oviduct, suggest that the hormonal regulation of PAI-1 in the isthmus may be different from other segments or isthmic PAI-1 may be more sensitized to circulating ovarian steroid concentrations during the estrous cycle. Progesterone, which peaks near Day 12 in the pig, was found to increase oviductal PAI-1 mRNA and may be the reason for elevated levels on Day 12 in the isthmus. A proposed function for elevated expression of PAI-1 mRNA on Day 12 in the isthmus is remodeling of oviductal ECM. No differences could be detected in expression of PAI-1 mRNA between pregnant and cyclic gilts.

Although direct evidence for an association between the presence of an early cleavage-stage embryo within the oviduct and changes in gene expression or protein synthesis by this tissue has not been demonstrated in the pig, some evidence suggests that the oviduct may respond to the presence of an embryo, as reviewed by Hunter (1988). Recently, the presence of embryos in the oviduct has been shown to regulate endometrial receptivity and increase the rate of implantation, suggesting communication between the early oviductal embryo and the maternal system [Wakuda et al., 1999].

The present data show that *de novo* protein synthesis and secretion of PAI-1 varies between segments and in the isthmus during early pregnancy. Higher levels of PAI-1 protein in the isthmus compared to the infundibulum or ampulla appears to mimic protein synthesis and secretion of oviductal TIMP-1 [Buhi et al., 1996a], suggesting a differential expression between the oviductal segments in expressing these protease inhibitors. Interestingly, PAI-1 protein secretion in culture media and oviduct flushes was found to be greater on Day 2 of early pregnancy coinciding with the time of fertilization and early embryonic cleavage-stage development. Elevated levels of isthmus PAI-1 and TIMP-1 on Day 2 suggest an important spatial and temporal role for these protease inhibitors. In addition, pig oviduct flushes contain a greater amount of plasminogen activator activity on Day 2 of early pregnancy than other days examined (as detailed in Chapter 5). Thus, greater concentrations of PAI-1 in the oviduct on Day 2 of early pregnancy, may be in response to the increased protease activity within the oviduct or associated with the early cleavage-stage embryo. Mouse and rat pre-implantation embryos have been shown to have uPA [Zhang et al, 1994, Harvey et al, 1995] and tPA activity [Zhang et al, 1992, Carroll et al, 1993], hence, the presence of PAI-1 may be

required to regulate the discrete focal requirements of the PAs. The protease inhibitors, PAI-1 and TIMP-1, may tightly regulate the remodeling and degradation of the ECM within the oviduct or early cleavage-stage embryo. Potential functions might be to prevent adhesion and implantation or protect the extracellular matrix of the zona pellucida from digestion by proteolytic enzymes. Serine protease inhibitors have been shown to stimulate DNA synthesis in endometrial glandular epithelial cells of pregnant pigs [Badinga et al., 1999] while TIMP-1 was found to increase the *in vitro* development of pig [Funahashi et al., 1997] and cow [Sato et al., 1994] blastocysts. PAI-1 may be having similar functions on DNA synthesis and/or development of the pre-implantation embryo.

Breed-dependent variations (Large White vs. Meishan) in expression of PAI-1 in the isthmus may be important relative to differences observed in time and synchrony of estrus and ovulation between the two breeds [Martinat-Botte et al., 1989, Terqui et al., 1990, Faillace et al., 1991, Wilmut et al., 1992, Hunter, 1993a]. Hunter et al. (1993b) suggests that the chronology of events within the estrus period may be critical to the recognized prolificacy of Meishan pigs. However, the presence of greater PAI-1 protein synthesis and secretion by the isthmus on Day 0 of pregnancy in Meishan gilts is difficult to explain. As reviewed by Hunter et al. (1993b), Meishan pigs were shown to have higher levels of circulating estradiol, greater estrogen concentrations in preovulatory follicles, and greater aromatase activity than the Large White. Therefore, it is unclear how the Chinese Meishan gilt secretes a greater level of PAI-1 under an elevated estrogen environment, as estrogen was found to decrease PAI-1 protein synthesis and secretion in

crossbred gilts. This would suggest the possibility of other PAI-1 regulatory factors or breed differences in hormonal regulation.

Oviductal PAI-1 appears to be regulated by changing levels of circulating ovarian steroid hormones, estrogen and progesterone. Synthesis and secretion of PAI-1 *in vitro* was inhibited in tissue cultured from OVX estrogen-treated gilts, however, this inhibition could be relieved when progesterone was given in combination with estrogen. Similarly, progesterone-mediated stimulation of PAI-1 mRNA levels could be inhibited when estrogen was given in combination with progesterone. This data suggests that regulation of transcription, synthesis, and secretion pathways for PAI-1 by estrogen or progesterone is dependent upon the presence or absence of the inhibitory steroid. Additionally, estrogen and progesterone may be temporally regulating these pathways through genomic and non-genomic actions. Progesterone has been shown to increase PAI-1 mRNA in endometrial stromal cells [Casslen et al., 1992, Schatz and Lockwood, 1993] and a synergistic stimulation of PAI-1 mRNA has been described for estrogen and progesterone in the endometrium [Schatz et al., 1994]. Immunolocalization of PAI-1 in secretory granules in the isthmus at Day 0 (high estrogen) and Day 9 (elevated progesterone) [Chapter 3], suggested that while estrogen decreases PAI-1 protein synthesis and secretion, it does not inhibit its accumulation and storage in secretory granules. Thus, this supports our present data that estrogen does not inhibit transcription of PAI-1 mRNA. While various *in vitro* studies have shown progesterone stimulates expression of PAI-1 mRNA and protein [Schatz and Lockwood, 1993, Casslen et al., 1992] our *in vivo* study utilizing OVX animals was able to show a direct effect of this steroid on protein secretion only in the presence of estrogen. Within the oviduct, we postulate that other regulatory

factors may be interacting *in vivo* to control PAI-1 synthesis and secretion. Regulators of uterine PAI-1 mRNA and/or protein include, epidermal growth factor (EGF) [Miyachi et al., 1995] and transforming growth factor-beta (TGF β) [Bruner et al., 1995, Graham, 1997]. These two growth factors have been immunolocalized in the pig oviduct [Buhi et al., 1997] and measured in oviductal fluid [Swanchara et al., 1995, Buhi et al., 1997]. PAI-1 has also been shown to be regulated in extravascular tissues by gonadotropins, cytokines, and dexamethasone, [Andreasen et al., 1990].

In summary, the presence of elevated levels of PAI-1 mRNA and protein in the oviduct on Day 2 of early pregnancy coinciding with fertilization and subsequent early cleavage-stage embryonic development, suggest that PAI-1 might play an important role in facilitating or regulating one or both of these events. Additionally, the circulating ovarian steroids, estrogen and progesterone, were shown to regulate the expression of oviductal PAI-1 mRNA and protein in the oviduct.

CHAPTER 5

PLASMINOGEN ACTIVATOR INHIBITOR-1 (PAI-1) AND uPA ACTIVITY IN THE OVIDUCT AND ASSOCIATION OF PAI-1 WITH THE PREIMPLANTATION EMBRYO

Introduction

The plasminogen/plasmin system has recently been shown to be an important component of several proteolytic cascades which occur during the process of fertilization [Huarte et al., 1993]. Well-characterized proteolytic events during fertilization include the acrosome reaction by spermatozoa and digestion through the zona pellucida matrix, as well as the cortical granule reaction leading to the zona block to polyspermy [Yanagimachi, 1988]. However, besides acrosin, the proteolytic enzymes and their inhibitors are relatively unknown. The zymogen, plasminogen, is relatively abundant in uterine fluid [Finlay et al., 1983], ovarian follicular fluid [Beers, 1975] and seminal plasma [Zaneveld et al., 1975, Kobayashi et al., 1992]. Ovulated eggs have been shown to secrete tissue plasminogen activator (tPA) [Huarte et al., 1985, Zhang et al., 1992] and urokinase plasminogen activator (uPA) is associated with ejaculated spermatozoa in the mouse [Huarte et al., 1987], human [Smokovitis et al., 1992] and pig [Smokovitis et al., 1992]. Although the exact nature of PA involvement during fertilization is unknown, indirect evidence suggests that these enzymes, uPA and tPA, may have a role in spermatozoal penetration through the zona pellucida. Male mice homozygous for a targeted mutation in the acrosin gene remain fertile and spermatozoa penetrate the zona

pellucida in the complete absence of acrosin activity [Baba et al., 1994, Adham et al., 1997]. Huarte et al. (1993) showed that addition of plasminogen during *in vitro* fertilization in the mouse increased the percentage of eggs fertilized, while antibodies that inhibit the catalytic activity of plasmin decreased fertilization. Plasminogen activators have also been found to be associated with ovine [Bartlett and Menino, 1993] and bovine embryos [Berg and Menino, 1992] and uPA mRNA is found in 2-cell rat embryos to the blastocyst stage [Zhang et al., 1994].

The inhibition of the active uPA and tPA enzymes is an important element in control of the plasminogen/plasmin system. Both uPA and tPA convert the proenzyme plasminogen into the broad specificity enzyme plasmin. Plasminogen activator inhibitor-1 is the primary physiological inhibitor of both uPA and tPA and is present in a wide variety of tissues and cultured cells [Andreasen et al., 1992]. PAI-1, a glycoprotein, consists of 379 amino acids, corresponding to a 43 kDa polypeptide chain [Andreasen et al., 1992], and is a member of the serine protease inhibitor superfamily (serpins). This family, which includes the majority of plasma protease inhibitors, has been shown to be involved in fibrinolysis, ovulation, and implantation [Andreasen et al., 1992]. An interesting feature of PAI-1 is that it exists in two forms - an active form which is synthesized and secreted by cells into culture media or the extracellular space, and an inactive or latent form [Hekman and Loskutoff, 1985]. PAI-1 converts spontaneously to the latent form shortly after secretion and can be subsequently reactivated by treatment with denaturants and negatively charged phospholipids [Andreasen et al., 1992]. However, the active PAI-1 is stabilized by its association with extracellular matrix [Mimuro and Loskutoff, 1989] or vitronectin in plasma [Declerck et al., 1988]. The

major *de novo* synthesized and secreted protein of the pig isthmus was identified as PAI-1 [Chapter 3]. This protein was localized to the apical region of oviductal epithelium and found to be associated with putative secretory granules of the isthmus. PAI-1 mRNA and protein were found to be greatest on Day 2 of early pregnancy in gilts, coinciding with the time of fertilization and early cleavage-stage embryonic development.

The temporal and spatial expression of PAI-1 in the oviduct coinciding with fertilization and early embryonic development suggests that this protease inhibitor may have an important function during these events. However, it is unknown if this protein retains biological activity after secretion into the oviductal lumen or if it associates with oviductal oocytes or embryos. The objectives of this study were 1) to characterize the biological activity of oviductal PAI-1 and its interaction with uPA, 2) to evaluate PA activity in the oviduct during early pregnancy, and 3) to examine PAI-1 association with oviductal oocytes and embryos.

Materials and Methods

Materials

All culture materials used are described in Chapters 3 and 4. Unless otherwise stated, all other chemical reagents and supplies were obtained from Sigma-Aldrich or Fisher Scientific.

Animals and Collection of Oviductal Flush

Sexually mature crossbred gilts (Yorkshire X Duroc X Hampshire) were observed daily for behavioral estrus for at least two estrous cycles in the presence of an intact boar. The first day of standing estrus was designated Day 0. Gilts were bred at the onset of estrus and again 24 h later to ensure fertile mating (except animals assigned to Day 0 of

pregnancy). Reproductive tracts were collected aseptically after sacrifice at the local abbatoir or after surgery. For surgery, anesthesia was induced with a combination of Telazol (Fort Dodge, IA, 2.2 mg/kg) and Xylazine (Fort Dodge, 2.2 mg/kg), administered intramuscularly. Anesthesia was maintained via inhalation of Halothane (Halocarbon Laboratories, River Edge, NJ)-oxygen mixture. Reproductive tracts were excised after midline laparotomy, following which the incision was closed, and gilts allowed to recover. Oviducts were flushed from the fimbria-infundibulum through the isthmus with 5 ml of (modified) Eagle's minimum essential medium (MEM) using a 10 ml disposable syringe and 20-gauge needle. Flushed material was collected into a sterile 15 ml conical tube and stored at -20° C. Animal-use protocols were approved by the University of Florida Institutional Animal Care and Use Committee.

Oviductal PAI-1/uPA Complex Formation

Semi-purified radiolabeled ($[^3\text{H}]$ -leucine) porcine PAI-1 was obtained from isthmus-conditioned oviduct explant culture medium after fractionation on a heparin-agarose affinity column chromatography as previously described in Chapter 3. Heparin-agarose fractions (0.2 M and 0.4 M NaCl elution) were resuspended in PBS (2 mg protein/1.5 ml; pH 7.4). Latent porcine PAI-1 was activated by incubation with 4 M guanidine-HCl for 2 h at room temperature, and then dialyzed against PBS (2 changes, 2 L each, 12 h each, 4° C). Non-activated PAI-1 was treated as above except for addition of 4 M guanidine-HCl (volume was adjusted with PBS). After dialysis, samples were counted for radioactivity by liquid scintillation spectrometry. To evaluate complex formation, activated and non-activated PAI-1 (29,000 cpm each) were incubated with or without 5 μg of human urokinase for 30 min at 37° C. Additionally, activated PAI-1

(29,000 cpm) was incubated with a polyclonal rabbit anti-human PAI-1 or normal rabbit sera (15 μ l each) followed by a 30 min exposure to urokinase as described. The purpose of addition of the antibody to the activated PAI-1 was to test for inhibition of complex formation. Samples were then run on a 10% non-reducing 1D-SDS-PAGE gel as previously described [Buhi et al., 1989].

Inhibition of uPA Activity by Oviductal PAI-1

Semi-purified PAI-1 was activated using guanidine-HCl as described above. To evaluate the amount of exogenous uPA activity that could be inhibited by oviductal-derived PAI-1 a direct peptidyl anilide assay was used according to Munch et al. (1993). To compare inhibition of uPA activity between activated or latent PAI-1, various amounts of activated or latent PAI-1 (0-40 μ g) were incubated with uPA (0.5 μ g/well) in PBS (pH 7.4) for 1.5 h at room temperature in a 96-well Nunc-immuno plate (Nalgene Nunc International, Rochester, NY) (final volume was 200 μ l). After incubation, 10 μ l of the chromogenic substrate S-2444 (0.4 mM) (Sigma-Aldrich, St. Louis, MO) was added to each well. Hydrolysis of the substrate to a tripeptide and the yellow p-nitroaniline (pNA) was determined by following the absorbance at 405 nm on a ThermoMax microplate reader (Molecular Devices, Menlo Park, CA). All samples were run in triplicate. Controls included uPA alone, substrate alone and PAI-1 alone (both activated and non-activated). Additionally, activated PAI-1 (40 μ g) was added to uPA + substrate after a 30 minute co-incubation in order to determine if activated oviductal-PAI-1 could inhibit the enzymatic reaction once initiated.

Oviductal Plasminogen Activator Activity During Early Pregnancy

To examine PA activity in oviductal flushes obtained from early pregnant gilts (Days 0, 1, 2, 5 and 12), a peptidyl anilide assay was employed using the chromogenic substrate, 2-44x (0.4 mM) (American Diagnostica, Greenwich, CT). Oviductal flushes were pooled by day in order to obtain enough antigen to accurately measure activity from each day. Two different pools were established from separate animals according to day and analysis are the means of two individual replicates. Pooled samples were centrifuged ($2,200 \times g$, 10 min, $4^{\circ}C$) and the supernatant dialyzed against dH_2O (three changes, 4 L each, 12 h each, $4^{\circ}C$). After dialysis, total protein was measured by the Bio-Rad protein assay according to manufacturer's instructions. Aliquots (950 μg) from each day of early pregnancy were lyophilized and stored at $-20^{\circ}C$. Samples were resuspended in 325 μl PBS (pH 8.8) and 100 μl of sample (300 μg protein) was added to a 96-well plate. After a 10 min incubation with 10 μl of 2-44x, hydrolysis and formation of pNA was followed over time (absorbance at 405 nm) on a ThermoMax microplate reader. All samples were run in duplicate, and PA activity expressed as optical density/ μg oviduct protein. Controls included oviductal protein alone and substrate alone. This experiment was replicated twice using two different oviductal flush pools. For number of animals within each pool/day, see Figure 5-5 legend.

Amiloride, an inhibitor of uPA but not tPA [Vassalli and Belin, 1987] was incorporated into the assay in order to determine which PA was being measured. Using a fixed concentration of exogenous uPA (0.5 μg), various concentrations of amiloride (0, 0.25, 0.5, 1.25, 2.50 and 5.00 mM) were tested for inhibitory activity in order to determine the appropriate level for the assay. Briefly, 10 μl of uPA was incubated with

100 μ l of amiloride and PBS, pH 8.8 (final volume, 200 μ l) for 30 min in a 96-well plate and substrate formation read at 405 nm. A concentration of 2.5 mM was shown to almost completely inhibit exogenously added uPA activity and was subsequently used in all inhibition assays. Oviduct flushes from Days 0, 2, 8 and 15 were pooled together, centrifuged, dialyzed, and lyophilized as described above. Aliquots (5 mg) were resuspended in 1 ml PBS (pH 8.8) and pulse-centrifuged (10,000 \times g) to remove insoluble material. Pooled oviduct flushes were incubated with or without amiloride (2.5 mM) for 10 minutes prior to addition of 2-44x (0.4 mM). The assay was run in a 96-well plate and absorbance (405 nm) recorded over time as described. The uPA activity in the pooled oviduct flush was compared to a standard curve (0-5 IU human uPA), allowing for estimation of the uPA concentration.

Association of Oviductal PAI-1 With the Oocyte or Embryo

Immunogold electron microscopy was utilized to localize PAI-1 in preovulatory follicular oocytes (Day 0), oviductal oocytes (Day 2), oviductal embryos (Day 2), and uterine embryos (Day 4). Two animals were used for each day and multiple (>3) oocytes or embryos were evaluated from each animal. Preovulatory follicles were aspirated with a sterile 10 ml disposable syringe and a 20-gauge needle. Oviductal oocytes and embryos were flushed from the oviduct as described. Uterine embryos were collected by clamping the bifurcation with a hemostat and the uterine horns flushed abovarian with 10 ml of MEM. Oocytes and embryos were collected from sterile watch glasses using a dissecting stereo-microscope, evaluated for cell number, and placed into fixative for 1 h in PBS, pH 7.4, containing 0.5% (v/v) glutaraldehyde, 4% (v/v) paraformaldehyde at 4^o C. Samples were submitted to the electron microscopy core (Interdisciplinary Center for

Biotechnology, University of Florida, Gainesville, FL) for processing within 1 h of collection. After fixation, oocytes and embryos were rinsed in PBS, dehydrated through a graded ethanol series, and embedded in Unicryl (British BioCell International, UK) under UV light at -10°C for 2 days. Thin sections (0.5 μm) were cut and collected on Formvar-coated 100 mesh nickel grids, and PAI-1 antigen detected by immunogold labeling. The polyclonal rabbit anti-human PAI-1 (kindly donated by Dr. Schleef, Scripps Institute, LaJolla, CA) and preimmune rabbit sera, diluted 1:1000 in a high salt Tween buffer (0.02 M Tris-HCl, 0.5 M NaCl, 1% [v/v] Tween 20, pH 7.2) supplemented with 1% (w/v) ovalbumin, were incubated overnight with grids in a humid chamber at 4°C . Sections were then incubated with a secondary antibody (goat anti-rabbit IgG, 1:30 dilution in PBS) conjugated to 18 nm colloidal gold (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA) for 1 h at room temperature. Sections were post-stained with 2% (w/v) uranyl acetate and Reynold's lead citrate. Grids were examined on a Hitachi H-7000 transmission electron microscope (Hitachi Scientific Instruments, Danbury, CT). Digital micrographs were taken on a Gatan BioScan/Digital Micrograph 2.5 (Gatan Inc, Pleasanton, CA). For antibody specificity, refer to Chapter 3.

Statistical Analysis

Data were analyzed by ANOVA using the General Linear Models procedure of the Statistical Analysis System (SAS Institute Inc., Cary, NC, 1988). Data are expressed as least-squares means \pm SEM and differences between treatment means evaluated using the student Newman-Kuels T-test. A probability of $p < 0.05$ was considered significant.

Results

PAI-1/uPA Complex Formation

To determine if oviductal-derived semi-purified radiolabeled PAI-1 could form a stable complex with uPA, reactivated and latent PAI-1 were incubated with exogenous uPA and complex formation evaluated using non-reducing 1D-SDS-PAGE and fluorography. Activated PAI-1 was able to form a complex with uPA as shown by the shift in the PAI-1 relative molecular weight and/or the formation of a previously described substrate-like cleavage product (42,000 M_r) [Urano et al., 1992] located just below PAI-1 (Figure 5-1). However, non-activated PAI-1 was unable to form a complex with uPA and generate a cleaved substrate-like product (Figure 5-1). These results indicate that complex formation between oviductal PAI-1 and uPA requires reactivation of PAI-1 by denaturants such as guanidine-HCl.

PAI-1 Inhibition of uPA Activity

The ability of activated or latent PAI-1 to inhibit uPA activity was evaluated using a peptidyl anilide assay as described in Materials and Methods. Semi-purified activated PAI-1 was able to inhibit uPA activity over time (8 h) at concentrations of 10-40 µg, while 1 µg had no inhibitory effect (Figure 5-2A). However, latent PAI-1 at all concentrations tested, was unable to inhibit uPA activity at any time during the incubation (Figure 5-2B). The inhibition by activated PAI-1 was shown to be dose-dependent with a greater inhibition of uPA activity at higher concentrations (15-40 µg protein) (Figure 5-3). Addition of activated PAI-1 30 min after exposure of uPA to S-2444 indicated that activated PAI-1 could specifically inhibit uPA activity after initiation of the enzymatic reaction (Figure 5-4).

PA Activity in the Oviduct During Early Pregnancy

To determine PA activity in the oviduct during early pregnancy, oviduct flushes were pooled by day and activity measured using the chromogenic substrate, 2-44X. An effect of day ($p < 0.01$) was detected for PA activity during early pregnancy, and was found to be greater on Day 2 than on other days examined (Figure 5-5). Days 5 and 12 showed the least amount of PA activity during early pregnancy. In order to determine if this activity was due to tPA or uPA, a specific inhibitor of uPA but not tPA was included in the assay. At a concentration of 2.5 mM, amiloride was found to be sufficient to inhibit an excess concentration of uPA (0.5 μg) (Figure 5-6). Treatment of pooled oviduct flushes (Days 0, 2, 8, and 15) with amiloride inhibited 73% of the total PA activity (9.5 h after initiation of reaction) (Figure 5-7). This indicates that the majority of PA activity in the oviduct is uPA while the remaining activity may be due to tPA.

PAI-1 Association with Oocytes and Embryos

Association of PAI-1 with oocytes or embryos was examined using immunogold electron microscopy (EM) to determine site-specific localization. Figure 5-8 is an electron micrograph of a preovulatory follicular oocyte surrounded by cumulus cells. Oocytes retrieved from preovulatory follicles showed a sparsity of PAI-1 located throughout the zona pellucida and perivitelline space. The oocyte as well, showed an infrequent scattering of PAI-1 throughout the cytoplasm (Figure 5-9). The compact mass of cumulus cells that surrounded the follicular oocytes had an intense nuclear localization of PAI-1, while the cytoplasm was only moderately stained (Figure 5-9). Oviductal oocytes, flushed from the oviduct on Day 2 of the estrous cycle, had a moderate level of PAI-1 localized throughout the zona pellucida, perivitelline space, and cytoplasm of the

oocyte (Figure 5-10). Oocytes retrieved from Day 2 gilts, had a high density of gold particles were associated with the outer edge of the zona pellucida (Figure 5-10). Oviductal embryos (2-4 cell) showed very similar localization of PAI-1 to that of oviductal oocytes. PAI-1 was densely associated with the outer rim of the zona pellucida and moderately localized throughout the interior of the zona matrix, perivitelline space, and blastomere cytoplasm (Figure 5-11). PAI-1 was also densely associated with the head region of boar spermatozoa bound to or embedded within the zona pellucida in these embryos (Figure 5-11). However, the sperm midpiece or tail showed no localization of PAI-1. Embryos (8-16 cell) having traversed through the isthmus, the primary site of PAI-1 synthesis and secretion, flushed from the tip of the uterine horn on Day 4 of early pregnancy, showed localization patterns similar to those of oviductal embryos. PAI-1 was densely associated with the outer surface portion of the zona pellucida and moderately associated throughout the zona pellucida matrix, perivitelline space, and blastomere cytoplasm (Figure 5-12). Spermatozoa, still attached to the zona pellucida were shown to have PAI-1 localized along the head region, but not detected on the midpiece or tail (Figure 5-12). Controls for localization of PAI-1 in association with preovulatory follicular oocytes, oviductal oocytes and embryos, or uterine embryos are shown in Figure 5-13.

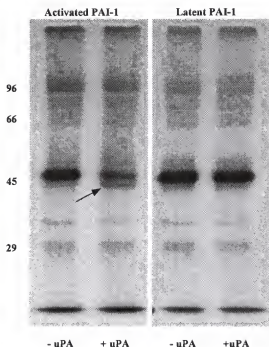
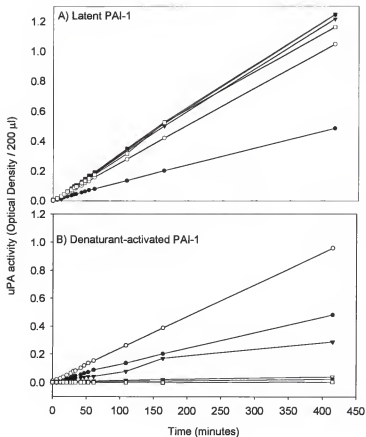


Figure 5-1. Representative fluorograph of denaturant-activated or latent PAI-1 incubated in the presence of uPA after non-reducing 1D-SDS-PAGE separation.

Activated PAI-1 (Lanes 1 and 2) or latent PAI-1 (Lanes 3 and 4) were incubated with (+) or without (-) 5 μ g of uPA as described in Materials and Methods. Activated PAI-1 incubated with uPA resulted in a complex formation and the generation of a substrate-like cleavage product (arrow) just below the 45,000 molecular weight marker ($\times 10^3$). Activated PAI-1 not exposed to uPA did not show a substrate-like cleavage product, nor did latent PAI-1 incubated with uPA.

Figure 5-2. Oviductal PAI-1 inhibition of uPA activity over time.

Various concentrations (0-40 μ g protein) of semi-purified latent (A) or activated (B) PAI-1 were incubated over time in the presence of uPA and S-2444 (chromogenic substrate). Generation of the chromophore, p-nitroaniline, was recorded at 405 nm absorbance and is expressed as relative uPA activity. Latent PAI-1 at all concentrations tested was unable to inhibit uPA activity while activated PAI-1 inhibited uPA at concentrations greater than 1 μ g. All samples were run in triplicate.



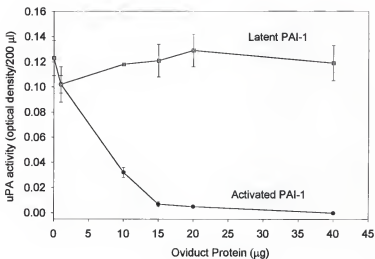


Figure 5-3. Dose-dependent inactivation of uPA activity by activated PAI-1.

Increasing concentration of semi-purified activated PAI-1 (1-40 µg) was shown to decrease relative uPA activity. Latent PAI-1 was unable to inhibit uPA activity. All samples were run in triplicate and absorbance (405 nm) was determined at 45 min after addition of S-2444.

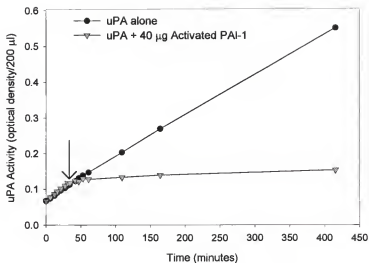


Figure 5-4. Inhibition of uPA activity with activated PAI-1 (40 µg) 30 minutes after initiation of the enzymatic reaction (cleavage of the chromogenic substrate, 2-44x, by uPA).

Absorbance (405 nm) was determined over time and all samples were read in duplicate. Arrow indicates point of addition of activated PAI-1. PAI-1 was able to completely inhibit the enzymatic cleavage of 2-44x by uPA.

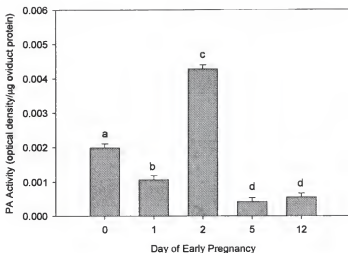


Figure 5-5. Plasminogen activator activity in oviduct flushes from early pregnant crossbred gilts.

Oviduct flushes were pooled (1 flush/animal/oviduct) randomly by day (0, 1, 2, 5, and 12; $n = 2, 5, 5, 3,$ and 4 animals/day respectively) and measured for PA activity using a peptidyl anilide assay as described in Materials and Methods. Values shown are least-squares means \pm SEM and this experiment was replicated twice using different pools for each day. Samples from each replicate were run in duplicate. An effect of day ($p < 0.05$) was determined for PA during early pregnancy. Bars with different superscripts are significantly different.

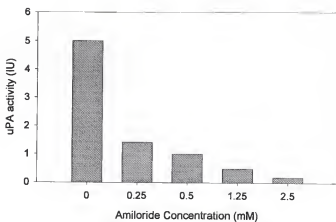


Figure 5-6. Dose-dependent inhibition of uPA activity by amiloride.

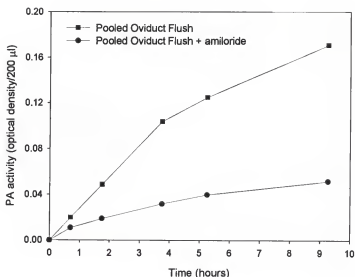


Figure 5-7. Inhibition of oviductal flush PA activity by amiloride.

Oviduct flushes from Days 0, 2, 8, and 15, were pooled together and PA activity was measured using a peptidyl anilide assay as described in Materials and Methods. Oviduct flushes were incubated with or without amiloride (2.5 mM) for 10 min prior to addition of 2-44x. All samples were run in duplicate and absorbance (405 nm) recorded over time.

Figure 5-8. Electron micrograph of a pig preovulatory follicular oocyte with surrounding cumulus cells.

Cumulus cells surrounding the oocyte are shown by an arrow. OC (oocyte), ZP (zona pellucida).

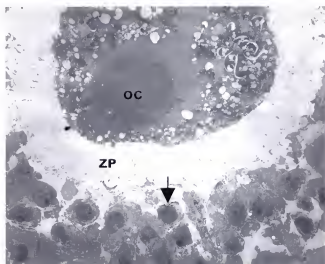


Figure 5-9. Immunogold localization of PAI-1 in association with pig preovulatory follicular oocytes and cumulus cells.

Gold particles (marked by arrows) can be seen primarily in the nucleus (nu) of cumulus cells (Panel A) and associated with the perivitelline space (PS) and oocyte (OC) (Panel B). Controls (NRS) are shown in Panel C (cumulus cells) and Panel D (PS + OC).

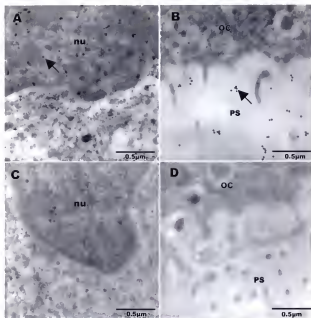


Figure 5-10. Immunogold localization of PAI-1 in association with oviductal oocytes.

Gold particles (marked by arrow) can be seen densely associated with the outer surface portion of the zona pellucida (ZP) (Panels A and B). Gold particles were also seen associated with the perivitelline space (PS) and oocyte (E should be OC; typing error) (Panel C) and within the oocytes cytoplasm (Panel D).

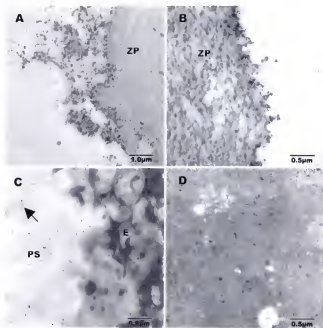


Figure 5-11. Immunogold localization of PAI-1 in association with oviductal embryos (2-4 cell) and spermatozoa attached and embedded in the zona pellucida.

Gold particles can be seen densely associated with the outer surface portion (arrow) of the zona pellucida (ZP) (Panel A) and also within the ZP matrix (Panel B). Additionally, gold particles (arrow) were associated with the perivitelline space (PS) and embryo (E) (Panel C). PAI-1 was also localized to the head region (arrow) of boar spermatozoa (Panel D).

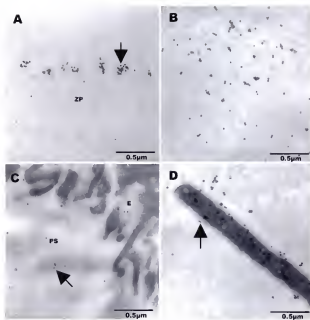


Figure 5-12. Immunogold localization of PAI-1 in association with early uterine embryos (8-16 cell).

Gold particles are seen densely associated with the outer surface portion (arrow) of the zona pellucida (ZP) (Panel A), moderately associated with the ZP matrix (Panel B), and embryo (Panel C). PAI-1 is also associated with the head region (arrow) of boar spermatozoa (Panel D) similar to oviductal embryos.

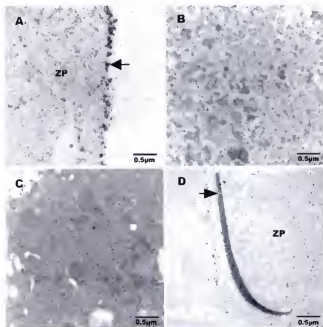
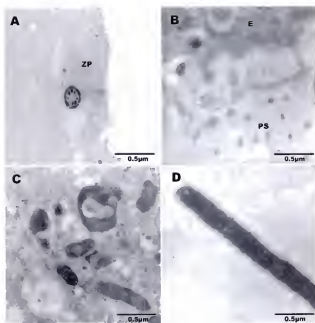


Figure 5-13. Representative controls (NRS) for immunogold localization of PAI-1 in association with follicular oocytes, oviductal oocytes, and embryos, and uterine embryos.

No gold particles were seen associated with the zona pellucida (ZP) (Panel A), perivitelline space (PS) (Panel B), embryo (Panel C), or spermatozoa (Panel D).



Discussion

This study demonstrates clearly that porcine oviductal-derived PAI-1, semi-purified from conditioned explant culture media, retains its biological activity and can interact with uPA. PAI-1, after activation with guanidine-HCl, bound to uPA and resulted in a shift in the molecular weight of PAI-1. Additionally, the interaction of activated PAI-1 with uPA resulted in formation of a cleaveable, substrate-like form (42,000 M_r) of PAI-1. PAI-1 preparations are heterogeneous and are a mixture of at least three different forms: active PAI-1 that can form complexes with PAs; latent PAI-1 which remains intact after incubation with PAs; and a population which is cleaved as a substrate by PAs [Urano et al., 1992]. Denaturant-activated PAI-1 can therefore act not only as an inhibitor but also as a substrate, and once exposed to catalytic amounts of PA is cleaved proteolytically in the reactive center [Munch et al., 1993]. This study demonstrates that radiolabeled oviductal PAI-1 can be purified from explant culture media and used for functional studies in the oviduct. Oviductal PAI-1 was able to inhibit the catalytic activity of uPA which was dependent upon the active or latent conformation. Non-activated (latent) PAI-1 fractions showed a much greater uPA activity than the exogenous uPA included in the assay. This is most likely due to the presence of endogenous PA in the semi-purified preparation. However, endogenous and exogenous PA activity are inhibited once PAI-1 is denaturant-activated. The activity of PAI-1 *in vivo* is likely stabilized by binding to the oviductal ECM or vitronectin [Declercq et al., 1988, Mimuro and Loskutoff, 1989]. However, the presence of vitronectin in the oviduct or its lumen has not been examined.

Plasminogen activator activity was identified in oviduct flushes throughout early pregnancy. Total PA activity was greatest in gilts on Day 2 of pregnancy that coincides with ovulation, fertilization, and early cleavage-stage embryonic development. The source of PA is unknown, although it could be from one of the three oviductal segments and/or selective serum transudate. Inhibition studies using a specific inhibitor of uPA, amiloride, revealed that uPA and likely some tPA are present in oviductal luminal fluid, however, the majority of this activity apparently belonged to uPA. Due to protein requirements to examine PA activity, the ratio of uPA/tPA on individual days of early pregnancy was not measured. The presence of PA in the oviduct and the possibility of plasminogen/plasmin involvement in fertilization [Huarte et al., 1993] and early embryonic development indicates the potential need of regulatory elements such as PAI-1. Interestingly, oviductal flush and culture media PAI-1 were greater on Day 2 of early pregnancy as described in Chapter 4. Therefore, increased expression and synthesis of PAI-1 mRNA, protein, and PA activity on Day 2 of early pregnancy compared to other days examined, suggest an important role in the regulation of the plasminogen/plasmin proteolytic cascade in the oviduct.

To the author's knowledge this study is the first demonstration of PAI-1 in association with oviductal oocytes and embryos. There were no observed differences in PAI-1 localization between oviductal oocytes and embryos, as both showed PAI-1 to be moderately localized throughout the interior of the zona pellucida, perivitelline space, and cytoplasm of the oocyte or blastomeres. The outer portion of the zona pellucida matrix in both oviductal oocytes and embryos had a high density of gold particles, suggesting that PAI-1, originating from the oviduct, coated the exterior of the zona

pellucida. Interestingly, PAI-1 was found to be densely associated with the head region of boar spermatozoa. PAI-1 has been shown to be in association with human spermatozoal membranes and boar spermatozoal membranes were shown to contain uPA and tPA [Smokovitis et al., 1992]. The localization of PAI-1 on spermatozoa here may be due to; 1) the presence of oviductal PAI-1 bound to spermatozoa, 2) endogenous PAI-1 associated with spermatozoal membranes, or 3) oviductal or testicular PAI-1 complexed to either tPA or uPA. Additionally, vitronectin, which associates with human spermatocytes, spermatids [Nuovo et al., 1995] and spermatozoa [Fusi et al., 1994, Bronson and Preissner, 1997], binds PAI-1 and stabilizes its activity both *in vitro* and *in vivo* [Declerck et al., 1988]. Evidence suggests that oolemal integrins and their ligands on spermatozoa play an integral role in gamete recognition, binding and subsequent fertilization [Sueoka et al., 1997]. As reviewed by Sueoka et al. (1997), numerous integrins have been identified on the oolema including $\beta_5\alpha_v$ and $\beta_6\alpha_v$, both of which bind vitronectin. Vitronectin is located in the acrosomal region of ejaculated spermatozoa and is liberated into culture medium following induction of the acrosome reaction by ionomycin [Fusi et al., 1994]. These findings, in addition to our data, suggest that oviductal PAI-1 may be bound to vitronectin on the spermatozoa. Therefore, PAI-1 is positioned to play a strategic role in gamete interactions leading to fertilization such as sperm binding to the zona pellucida, penetration through the zona pellucida matrix, and/or binding and fusion with the oolema.

PAI-1, shown to be densely associated with the outer portion of the zona pellucida, may function to protect the zona pellucida matrix from proteolytic degradation. Oviductal oocytes and embryos had a greater resistance to proteolytic digestion by

pronase than were either follicular oocytes or uterine embryos [Broermann et al., 1989]. This suggests that protease inhibitors secreted by the oviduct bind to the zona pellucida and stabilize the matrix. Fertilization and early embryonic development occur in a protease-rich environment when PA activity is greatest. In addition, numerous proteolytic enzymes are released from spermatozoa following the acrosome reaction. Thus, PAI-1 in the outer rim of the zona may help regulate proteolytic events to specific focal areas such as spermatozoa penetration of the zona pellucida matrix.

Supplementation of culture medium with proteases has been shown to improve the hatching rate of mouse embryos *in vitro* [Lee et al., 1997], and proteases are implicated in hatching and zona lysis [Perona and Wassarman, 1986, Menino and Williams, 1987, Gonzales and Bavister, 1995]. PAI-1 may act to stabilize the zona matrix and prevent premature hatching prior to blastocyst development. Plasminogen activators are secreted by oocytes following fertilization or by the early cleavage-stage embryo [Zhang et al., 1992, 1994] and are present in the oviductal microenvironment. These data suggest a regulatory molecule in the oviduct, such as PAI-1, may be required to prevent digestion of the zona pellucida matrix and premature hatching.

While follicular oocytes had sparse PAI-1, they did not have a dense coat of PAI-1 encircling the zona pellucida as seen in oviductal oocytes or embryos. This suggests that the heavy concentration of PAI-1 on oviductal oocytes and embryos was obtained from oviductal secretions. Cumulus cells were found to contain a high density of PAI-1 within their nucleus, suggesting PAI-1 associated with the follicular oocyte may be of cumulus cell origin. PAI-1 and PAI-2 mRNA has been detected in human cumulus and granulosa-luteal cells [Piquette et al., 1993] while PAI-1 mRNA has been detected in the

cow cumulus-oocyte complex [Bieser et al., 1998]. Uterine embryos (8-16 cell) collected on Day 4 of early pregnancy had a localization of PAI-1 similar to oviductal embryos. This suggests that the oviductal embryo may retain oviductal-derived PAI-1 in the uterine environment and did not dissociate from the embryo. However, it cannot be ruled out that this PAI-1 associated with the embryo is not of uterine origin.

In summary, functionally active PAI-1 is secreted by the oviductal epithelium at the time of ovulation, fertilization, and early cleavage-stage embryonic development. Active PAI-1 can then bind to and regulate the catalytic activity of plasminogen activators, such as uPA, which are present in the oviductal microenvironment. Additionally PAI-1 can associate with oviductal oocytes and embryos and may regulate the processes of sperm binding and/or penetration through the zona pellucida matrix. This process is possibly facilitated through the PAI-1 binding protein, vitronectin, that has been found associated with spermatozoa. Additional functions of PAI-1 may involve stabilization of the zona pellucida matrix to prevent proteolytic degradation or premature hatching.

CHAPTER 6
EFFECTS OF A PORCINE OVIDUCT-SPECIFIC GLYCOPROTEIN ON
FERTILIZATION, POLYSPERMY AND EARLY EMBRYONIC DEVELOPMENT IN
VITRO

Introduction

The porcine oviduct, responds to ovarian steroid hormones by synthesizing and releasing several specific proteins into the lumen [Buhi et al., 1990]. The cumulative synthesis and transport of these proteins into the oviductal lumen during proestrus, estrus and metestrus creates a microenvironment capable of supporting important reproductive events which include fertilization and early cleavage-stage embryonic development. Several *de novo* synthesized proteins of the porcine oviduct have been identified and characterized [Buhi et al., 1997], including the pig OSP. This protein is highly conserved across species including the human [Arias et al., 1994], sheep [DeSouza and Murray, 1995], mouse [Sendai et al., 1995], cow [Sendai et al., 1994], hamster [Suzuki et al., 1995], baboon [Donnelly et al., 1991] and rhesus monkey [Verhage et al., 1997]. Ovariectomy and steroid hormone replacement studies have shown that pOSP mRNA and protein synthesis are estrogen-dependent and that expression is greatest during the preovulatory period and at ovulation [Buhi et al., 1996]. The function of this protein remains unknown. Immunolocalization studies in the pig, however, have revealed pOSP to associate with the zona pellucida, perivitelline space and vitelline and blastomere

membranes of ovulated oocytes and oviductal embryos, respectively [Buhi et al., 1993], suggesting a role for this protein during fertilization and early embryonic development.

Embryo *in vitro* culture systems have been shown to result in successful *in vitro* maturation/fertilization (IVM/IVF) and subsequent development of the early cleavage-stage pig embryo to the blastocyst stage [Abeydeera and Day, 1997, Abeydeera et al., 1998a, Abeydeera et al., 1998b, Abeydeera et al., 1999]. However, a high incidence of polyspermy (often >50%) remains a major impediment in porcine IVF [Wang et al., 1991, 1994, 1997, Abeydeera and Day, 1997]. Differences in polyspermy rates have been shown to be much greater with *in vitro* matured oocytes (65%) than for ovulated oocytes flushed from the porcine oviduct and fertilized *in vitro* (28%) [Wang et al., 1998]. Co-culture of oocytes with oviductal epithelial cells [Kano et al., 1994] or preincubation of oocytes with oviductal fluid [Kim et al., 1996] significantly reduced the incidence of polyspermy in pigs. A functional block to polyspermy *in vivo* has been suggested to be due to a factor of oviductal origin. Several investigators have speculated that this activity may be due to a specific function of the OSP [Hunter, 1991, Dubuc and Sirard, 1995, Kim et al., 1996, Wang et al., 1998]. The association of pOSP with ovulated oocytes and early cleavage-stage embryos [Buhi et al., 1993] suggests this glycoprotein may be a likely candidate for such an activity. Funahashi and Day (1997) speculated that oviductal proteins in preincubation and/or fertilization media compete with sperm receptors for binding of zona pellucida ligands, stimulate the rate of sperm acrosome reaction and, thus, reduce the number of capacitated spermatozoa attaching to the surface of pig oocytes.

Specific objectives of this *in vitro* study were to; 1) evaluate the effects of pOSP on fertilization responses including penetration rate and the incidence of polyspermy, 2) examine the effect of pOSP on zona solubility and sperm binding to the zona, 3) determine if a polyclonal antibody generated against pOSP could negate specific pOSP effects, and 4) determine if pOSP has the capacity to enhance blastocyst development.

Materials and Methods

Materials

Acrylamide, N,N' diallyltartardiamide, urea, Nonidet P-40, and sodium dodecyl sulfate were acquired from Gallard-Schlesinger (Carle Place, NY); X-Omat AR film and photography reagents were a product of Eastman Kodak Co. (Rochester, NY); amino acids and protein standards were purchased from Sigma-Aldrich (St. Louis, MO); ampholines were from Pharmacia-Biotech (Piscataway, NJ); all other supplies and reagents for gel electrophoresis were procured from either Bio-Rad Laboratories (Richmond, CA) or Fisher Scientific (Orlando, FL); and all medium and culture supplies were obtained from Life Technologies (Grand Island, NY). L-[4,5-³H]leucine (specific activity, 120 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Unless otherwise stated, all other chemicals and reagents were acquired from Sigma or Fisher.

Culture Media

Basic *in vitro* oocyte maturation (IVM) medium was protein-free Tissue Culture Medium 199 [Abeydeera et al., 1999] supplemented with 10 ng/ml epidermal growth factor, 0.57 mM cysteine, 0.1% (w/v) polyvinyl alcohol (PVA), 0.5 µg/ml leutenizing hormone (LH), 0.5 µg/ml follicle stimulating hormone (FSH), 75 µg/ml potassium penicillin G, and 50 µg/ml streptomycin sulfate, 3.05 mM D-glucose, and 0.91 mM

sodium pyruvate. IVF medium was essentially that of Abeydeera and Day [1997], designated modified Tris-buffered medium (mTBM), pH 7.2-7.4 at 39°C, 5% CO₂ (v/v) in air. The mTBM consists of 113.1 mM NaCl, 3 mM KCl, 7.5 mM CaCl₂·2H₂O, 20 mM Tris (crystallized free base; Fisher Scientific, Fair Lawn, NJ), 11 mM glucose, 5 mM sodium pyruvate, and no antibiotics. Embryos were cultured in North Carolina State University (NCSU) 23 medium [Petters and Wells, 1993], supplemented with 4 mg/ml BSA (designated IVC medium). Medium (IVM, IVF and IVC) were covered with paraffin oil and equilibrated at 39°C, 5% CO₂ (v/v) in air at least 12 h prior to use.

In Vitro Maturation and *In Vitro* Fertilization

Ovaries from prepubertal gilts were collected from an abattoir and immediately transported to the laboratory at 25-30°C in 0.9% saline containing 75 µg/ml potassium penicillin G and 50 µg/ml streptomycin sulfate. Oocytes were aspirated from follicles (3-6 mm diameter) using a 20-gauge needle connected to a 10-ml disposable syringe, transferred to a 50 ml conical tube and allowed to sediment at room temperature (25°C). Supernatant was discarded and follicular contents were washed with Tyrode's Lactate (TL)-Hepes medium supplemented with 0.01% (w/v) PVA (TL-Hepes-PVA). Oocytes with an evenly granulated cytoplasm and surrounded by compact cumulus cells were washed twice with TL-Hepes-PVA, and three times in IVM medium. Oocytes (50-70) were suspended in 500 µl of IVM medium supplemented with LH and FSH in a 4-well multidish (Nunc, Roskilde, Denmark) and cultured for 42-44 h.

Upon completion of IVM, cumulus cells were removed by treatment with 0.1% (w/v) hyaluronidase in basic IVM medium and vortexed for 1 min. Denuded oocytes were washed three times in 500 µl of IVM medium followed by three washes in IVF

medium containing 1 mM caffeine and 1 mg/ml BSA. Oocytes ($n=35$) were placed into 50 μ l drops of the same medium, that had been covered with warm paraffin oil in a 35 x 10-mm² polystyrene culture dish (Becton Dickinson & Co., Lincoln Park, NJ) and pre-equilibrated (39°C, 5% [v/v] CO₂ in air) overnight. A frozen semen pellet was thawed and washed three times by centrifugation at 1,900 x g for 4 min in Dulbecco's PBS (DPBS; Life Technologies, Grand Island, NY) supplemented with 1 mg/ml BSA, 75 μ g/ml potassium penicillin G, and 50 μ g/ml streptomycin sulfate (pH 7.2). After washing, the sperm pellet was resuspended in IVF medium supplemented with caffeine (1 mM) and BSA (0.1%, w/v) and 50 μ l of the sperm suspension was added to 50 μ l drops of IVF medium containing the oocytes so that the final sperm concentration was 2.5-3.5 x 10⁵/ml. Spermatozoa and oocytes were coincubated for 6 h at 39°C in 5% CO₂ (v/v) in air.

Tissue Collection and Protein Purification

Sexually mature crossbred gilts (Yorkshire x Duroc x Hampshire) were observed daily for behavioral estrus for at least two estrous cycles in the presence of an intact boar. The first day of standing estrus was designated as Day 0 and animals were taken to the abattoir for slaughter on Day 0 or 1 of the estrous cycle. After exsanguination, reproductive tracts were collected aseptically, opened longitudinally, and oviductal tissue washed in several volumes of (modified) Eagles minimum essential medium (MEM). Tissue was cut into 1- to 3-mm³ sections and explants (500 mg) were cultured in 15 ml of leucine-deficient MEM containing 100 μ Ci of [³H]-leucine in petri dishes on a rocking platform at 39°C under a defined atmosphere of 50% N₂ (v/v): 47.5% O₂ (v/v): 2.5% CO₂ (v/v) [Buhi et al., 1990]. Non-labeled cultures were generated as above in complete

MEM, without addition of [^3H]-leucine. After 24 h of culture, media was aspirated and frozen at -20°C until purification.

Recent molecular cloning and analysis of porcine pOSP cDNA revealed a putative heparin-binding consensus sequence in the protein [Buhi et al., 1996]. Utilizing a heparin-agarose affinity column, highly purified pOSP was obtained (Alvarez and Buhi, unpublished). Briefly, culture media from Days 0 and 1 whole oviduct cultures were thawed, centrifuged ($2,200 \times g$, 10 min, 4°C), pooled, diluted (1:3) with 20 mM Tris-HCl (pH 7.6, 4°C) containing 0.02% (w/v) NaN_3 and slowly loaded onto the heparin-agarose column (2.5×8.2 cm) at 4°C . Porcine OSP was eluted utilizing stepwise increments of NaCl (0.1-3.0 M) and purification was monitored by 2D-SDS-PAGE and fluorography as previously described [Buhi et al., 1990]. Purified preparations from the 0.4 M NaCl elution were utilized for all experiments detailed herein. Highly-purified pOSP was dialyzed against dH_2O (3 changes, 4 L each, 24 h each, 4°C), pooled, measured for total protein content by the Bio-Rad microassay (according to manufacturer's instructions) and radioactivity by liquid scintillation spectrophotometry. Pooled samples were lyophilized and stored at -20°C .

Experiments 1, 2, 3 and 4 utilized radiolabeled pOSP obtained from whole oviduct cultures as detailed above. Experiment 4, designed to evaluate pOSP effects on embryonic development to the blastocyst stage, used pOSP purified from the ampulla segment of the oviduct and was not labeled with [^3H]-leucine. Purification of ampulla-derived pOSP was identical to that of the whole oviduct. Protein (5 mg) from either pOSP preparation was resuspended in 3 ml of mTBM (39°C) for IVF or NCSU 23 (39°C) for IVC and total protein content was measured by the Bio-Rad protein assay

according to the manufacturer's instructions. Aliquots were stored at -20°C . Oviductal pOSP resuspended in IVF medium was evaluated by 2D-SDS-PAGE and fluorography [Buhi et al., 1990].

IgG Purification

Polyclonal anti-pOSP antiserum was prepared as described by Buhi et al. (1993). The IgG fraction was separated by ammonium sulfate precipitation and purified by diethylamino ethyl sepharose (DEAE-Sepharose, Pharmacia-Biotech, Piscataway, NJ) ion-exchange chromatography [Harlow and Lane, 1988]. The isolated IgG fraction was dialyzed against dH_2O (2 changes, 2 L each, 24 h each, 4°C), total protein content was measured as described and purity determined by 1D-SDS-PAGE. Aliquots containing $100\text{ }\mu\text{g}$ of IgG were lyophilized and stored at -20°C . A dose-response of radiolabeled pOSP ($10\text{ }\mu\text{g/ml}$) binding was evaluated with various concentrations of purified anti-pOSP IgG ($10\text{--}200\text{ }\mu\text{g/ml}$). Incubation was allowed to extend overnight at 39°C in order to mimic conditions established for IVF. Antibody-pOSP complex was precipitated using Protein A-Sepharose (1 h incubation, gentle rotation, room temperature) and centrifuged ($2,200 \times g$, 5 min). Protein A-Sepharose complexes and supernatant were analyzed by 1D-SDS-PAGE and fluorography [Buhi et al., 1989]. The pOSP antiserum was shown previously to cross-react with only a couple of other oviductal proteins released into culture [Buhi et al., 1993] and these proteins were not purified during heparin-agarose affinity purification (data not shown). The pOSP antiserum does not cross-react with any of the radiolabeled proteins shown in Figure 6-1 [Buhi et al., 1993].

Experiment 1: pOSP Effects on *In Vitro* fertilization

Pig oocytes, matured *in vitro*, were exposed to various concentrations of pOSP before and during IVF. *In vitro* matured oocytes, after 42-44 h of culture, were removed of cumulus cells as described, washed three times in IVF medium, and randomly assigned to each of six treatments. Oocytes (n=35/treatment) were placed into 50 μ l drops of IVF medium containing 0, 0.1, 1, 10, 50 or 100 μ g/ml pOSP, and preincubated for 4 h at 39⁰ C, 5% (v/v) CO₂ in air. Oocytes were then inseminated with spermatozoa (50 μ l, 2.5-3.5 $\times 10^5$ /ml) essentially halving the concentration of pOSP used during preincubation. Six hours after insemination, oocytes were washed three times in IVC medium (100 μ l) and incubated in IVC medium (100 μ l) for an additional 4 h. After incubation in IVC medium, sperm cells attached to the zona were removed by washing three times in TL-Hepes-PVA (39⁰ C) with a small bore pipette, mounted, and placed into fixative (25% [v:v] acetic acid in ethanol, room temperature) for 72 h. Oocytes were then stained with 1% (w:v) orcein in 45% (v:v) acetic acid and examined under a phase-contrast microscope at x200 and x400 magnification. The meiotic stage of the oocytes were assessed according to Hunter and Polge [1966]. Oocytes were considered penetrated when one or more sperm heads and/or male pronuclei and corresponding sperm tails were present. The rate of polyspermy, male pronuclei formation, and mean number of sperm/oocyte, are determined from those oocytes which are penetrated. Experiment 1 was replicated five times with multiple observations for each replicate.

Experiment 2: Anti-pOSP IgG Inhibition of the Decrease in Polyspermy

Preliminary experiments testing various concentrations of pOSP IgG (10, 50, 100, 200 and 400 μ g/ml) indicated that 50 μ g/ml of IgG could inhibit the effects seen on

polyspermy without affecting penetration rates. For this experiment, a concentration of 10 µg/ml pOSP was used. Treatments consisted of control (no addition), pOSP, pOSP + IgG (50 µg/ml), or antibody alone and were evaluated as a 2 x 2 factorial design. Cumulus-free oocytes were assigned randomly to each of the four treatments (n=35/treatment), preincubated for 4 hours, inseminated, and fertilization parameters evaluated as above. Experiment 2 was replicated 3 times with multiple observations within each replicate.

Experiment 3: pOSP Effects on Zona Pellucida Solubility

To test zona pellucida solubility, cumulus-free *in vitro* matured oocytes were washed three times in 500 µl drops of IVF medium and transferred to 50 µl drops of IVF medium containing various concentrations of pOSP (0, 0.1, 1, 10, 50 or 100 µg/ml) for 4 h at 39⁰ C, 5% (v/v) CO₂ in air. Oocytes were then washed three times in TL-Hepes-PVA (39⁰ C) and oocytes (n=15/treatment) placed into 100 µl of a 0.1% (w:v) pronase solution in phosphate-buffered saline (PBS). Zona digestion was observed continually at room temperature (25⁰C) with an inverted microscope. When the zona pellucida was no longer visible at 200x magnification, the zona pellucida dissolution time was recorded (total number of oocytes missing zona pellucidas at designated time points – see Table 6-2 for time points). Experiment 3 was replicated 3 times.

Experiment 4: Effects of pOSP on Sperm Binding to the Zona Pellucida

To examine for pOSP effects on sperm binding, cumulus-free *in vitro* matured oocytes (n=35/treatment) were washed and preincubated with various concentrations of pOSP (0, 0.1, 1, 10, 50 or 100 µg/ml) for 4 h at 39⁰ C and coincubated with spermatozoa as described above. After fertilization, putative zygotes were washed twice in 500 µl of

IVC medium and pipetted in and out (10 times) of a wide bore pipette to remove loosely bound sperm. Putative zygotes were then placed into 50 μ l drops of TL-Hepes-PVA containing Hoescht 33342 (bisBenzamide; 1.3 mg/ml) and incubated for 30 min at 39 $^{\circ}$ C in 5% (v/v) CO $_2$ in air. Putative zygotes were then washed twice in 300 μ l of TL-Hepes-PVA, mounted, and the number of tightly bound sperm/zygote counted under a phase-contrast microscope (Leitz Laborlux D) equipped with ultraviolet illumination (excitation at 330-380 nm, emission at 420 nm). Experiment 4 was replicated 3 times with 15 spermatozoa counted from each replicate.

Experiment 5: pOSP Effects on Cleavage Rate and Embryonic Development

In vitro matured oocytes were removed of cumulus cells, washed and randomly assigned to each of two treatments; control (0 μ g/ml of pOSP during preincubation/IVF, n=35 oocytes/50 μ l drop, n=2 drops) or pOSP (10 μ g/ml during preincubation/IVF, n=35 oocytes/50 μ l drop, n=10 drops). A concentration of 10 μ g/ml of pOSP was chosen because, compared to the control, this concentration significantly decreased the incidence of polyspermy yet had no effect on the penetration rate. Preincubation and fertilization were carried out as described. Six hours after fertilization, control putative zygotes were washed three times in 100 μ l drops of IVC medium, transferred to a fresh 100 μ l drop (n=35 oocytes/drop) and incubated at 39 $^{\circ}$ C in 5% (v/v) CO $_2$ in air. Putative zygotes exposed to pOSP during preincubation/IVF were pooled, washed three times in 500 μ l of IVC medium and randomly assigned to 100 μ l IVC drops containing 0, 1, 10, 50 or 100 μ g/ml of pOSP (n=70 zygotes/treatment, 35 embryos/drop). At 48 and 144 h after insemination, cleavage rate and blastocyst formation, respectively, were evaluated under

a stereomicroscope. Blastocyst formation was calculated from the number of oocytes. Experiment 5 was replicated 9 times with multiple observations within each replicate.

Statistical Analyses

Data were analyzed by ANOVA using the General Linear Models procedure of the Statistical Analysis System (SAS Institute Inc., Cary, NC, 1988). All percentage data were subjected to arcsine transformation before statistical analysis. Data are expressed as means \pm SEM or least squares means \pm SEM. Differences between means were evaluated by the Student Newman-Kuels T-test. The model for analysis included the main effects of treatment (0-100 μ g/ml pOSP), replicate, and replicate x treatment. Replicate and replicate x treatment was not significant in any of the analysis. Therefore, replicate was not included in the final model (main effect of treatment alone) as the analyses were no longer significant when included in the model. A probability of $p < 0.05$ was considered significant. A 2 x 2 factorial design was used to evaluate the effects of pOSP and anti-pOSP IgG on polyspermy. A set of pre-planned orthogonal contrasts were used to evaluate pOSP concentrations on embryonic development. Contrast one evaluated blastocyst development between oocytes exposed to 10 μ g/ml pOSP during preincubation/IVF and the control (0 μ g/ml pOSP). Contrast two evaluated oocytes exposed to pOSP during preincubation/IVF alone vs. those oocytes exposed to pOSP during preincubation/IVF and embryonic development. Contrast three evaluated the two lowest concentrations of pOSP added during embryo development (1 and 10 μ g/ml pOSP) vs. the higher concentrations of pOSP (50 and 100 μ g/ml).

Results

Purification of pOSP

A representative 2D-SDS-PAGE fluorograph of conditioned oviductal culture media fractionated using a heparin-agarose affinity column resulted in a highly purified preparation of pOSP (Figure 6-1). The 0.4 M NaCl elution showed an 80-85% purification of pOSP protein from oviductal culture media (Alvarez, Buhi, unpublished). Porcine OSP is the primary protein product that was resuspended in IVF medium. Several minor proteins from the oviduct that contain heparin-binding regions or are complexed with pOSP were found to be co-purified along with pOSP (not shown). The majority of these minor proteins appear not to be *de novo* synthesized products of the oviduct as they are not labeled with [³H]-leucine, suggesting that they are from serum transudate. Four fractions of pOSP were identified in the purified preparation: pOSP 1-3 previously identified [Buhi et al., 1990] and a fourth protein that concentrated during purification that can react with pOSP antibody (Buhi, Alvarez, unpublished). The radiolabeled proteins which are not pOSP (possibly PAI-1, see Chapter 3 fluorograph), shown in Figure 6-1, do not react with the pOSP antibody [Buhi et al., 1993].

Experiment 1: Effects of pOSP on *In Vitro* Fertilization

To examine pOSP effects on *in vitro* fertilization, IVM pig oocytes were preincubated with various concentrations of pOSP (0-100 µg/ml) and then fertilized in the presence of pOSP. Effects of pOSP on fertilization responses are shown in Table 6-1. A pOSP treatment effect ($p < 0.05$) was found for sperm penetration. Concentrations of pOSP from 0-50 µg/ml had no effect on penetration, while 100 µg/ml of pOSP significantly decreased sperm penetration of pig oocytes (41%) compared to control

(74%) (Table 6-1). A treatment effect ($p<0.05$) for pOSP also was determined on the incidence of polyspermy. Concentrations of pOSP from 10-100 $\mu\text{g/ml}$ significantly decreased polyspermy (24-29%) compared to control (61%) (Table 6-1). No effect of pOSP was determined on the formation of the male pronucleus. A treatment effect ($p<0.05$) with pOSP was also determined for the mean number of sperm (MNS)/oocyte. A concentration of 10 $\mu\text{g/ml}$ pOSP was selected for all subsequent experiments since this concentration significantly decreased polyspermy yet maintained penetration rates similar to control. A photograph of a polyspermic porcine oocyte is shown in Figure 6-2.

Experiment 2: Anti-pOSP IgG Inhibition of the Decrease in Polyspermy

To determine whether the decrease in polyspermy was due to a specific effect of pOSP and not an effector which copurified with pOSP, a polyclonal anti-pOSP IgG was included during preincubation/IVF. For cross-reactivity see IgG purification in Methods and Materials. Experiments testing various concentrations of IgG (10, 50, 100, 200 and 400 $\mu\text{g/ml}$) indicated that concentrations of 200 $\mu\text{g/ml}$ or greater reduced penetration of oocytes, adversely affecting polyspermy rates (data not shown). However, addition of an IgG concentration of 50 $\mu\text{g/ml}$ maintained a penetration and polyspermy rate similar to that of the control. Similar to the previous experiment (Table 6-1), an effect of pOSP ($p<0.05$) (Figure 6-3) was found on polyspermy and an interaction was detected for pOSP and anti-pOSP IgG ($p<0.05$), indicating that the effect of pOSP on polyspermy is dependent on the presence or absence of antibody. This result demonstrates that the pOSP-induced decrease in polyspermy rate was due to a specific effect of pOSP and not an effector molecule, if any, that co-purified with pOSP. Here, 10 $\mu\text{g/ml}$ pOSP decreased

the incidence of polyspermy (23%), while addition of specific pOSP antibody showed a polyspermy rate similar to that of the control (46%) (Figure 6-3).

Experiment 3: Effects of pOSP on Zona Pellucida Solubility

Effects of pOSP on zona pellucida solubility were examined to determine if enzymatic digestion of the zona pellucida was altered by pOSP association. When incubated for 4 hours in the presence of various concentrations of pOSP (0-100 µg/ml), pOSP concentrations tested failed to alter zona pellucida dissection time (Table 6-2).

Experiment 4: Effects of pOSP on Sperm Binding to the Zona Pellucida

To determine if the decrease in polyspermy *in vitro* is due to a change in the number of spermatozoa that are attached to the zona pellucida, pOSP effects on sperm binding were examined. The number of sperm bound to each zygote/treatment was evaluated and determined by fluorescent microscopy (Figure 6-5) as detailed in Materials and Methods. A treatment effect ($p < 0.05$) of pOSP on sperm binding was demonstrated. The number of sperm bound/zygote was significantly reduced with a decrease in sperm binding observed with increasing concentrations of pOSP (Figure 6-4). The decrease in the number of spermatozoa bound to the zona pellucida with increasing concentrations of pOSP appears to have reached a plateau by 1 µg/ml of pOSP.

Experiment 5: Effects of pOSP on Embryonic Development

To test whether pOSP had an effect on embryonic development *in vitro*, pOSP was included during preincubation/IVF alone or during preincubation/IVF + IVC and both the cleavage rate and development to blastocyst examined. There were no treatment effects observed on cleavage rates of oocytes fertilized and cultured in the presence of pOSP (Figure 6-6). There was, however, a treatment effect of pOSP ($p < 0.05$) on

development of embryos to blastocysts. A significant increase ($p<0.05$) in blastocyst number was found when pOSP was included during preincubation/IVF compared to control (Figure 6-7). No additional effect of pOSP (1, 10, 50 or 100 $\mu\text{g/ml}$) added during IVC could be detected. Higher concentrations of pOSP (50 and 100 $\mu\text{g/ml}$) during IVC tended ($p=0.08$) to decrease the effect observed on development to blastocyst when pOSP was added during preincubation/IVF, however, the blastocyst development was still above those of the control.

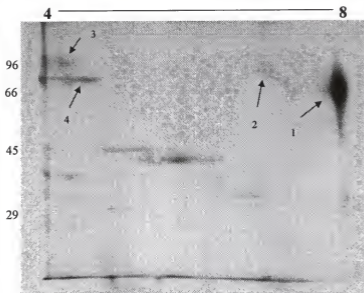


Figure 6-1. Representative fluorograph of [^3H]-lucine labeled proteins (500 μg) from whole oviduct explant-conditioned culture media (Day 0/1) subjected to heparin-agarose affinity column chromatography and separated by 2D-SDS-PAGE.

Lyophilized proteins were resuspended in IVF media prior to electrophoresis. Porcine OSP (1-4) are marked by arrows. Molecular weight markers ($\times 10^3$) are indicated and the pH gradient runs from left (pH 8) to right (pH 4)

Table 6-1. Effect of pOSP on Fertilization Parameters of Pig Oocytes Matured and Fertilized *In Vitro*.

pOSP ($\mu\text{g/ml}$)	No. oocyte	Penetrated (%)	Polyspermic (%)	(%) Male Pronucleus	MNS/ Oocyte
0.0	160	74.4 ± 5.2^a	61.2 ± 4.0^a	92.0 ± 3.5^a	1.9 ± 0.11^a
0.1	168	59.4 ± 4.8^{ab}	49.6 ± 7.0^a	88.0 ± 4.3^a	1.8 ± 0.20^{ab}
1.0	176	67.4 ± 5.3^{ab}	46.6 ± 6.8^a	91.8 ± 1.1^a	1.7 ± 0.16^{ab}
10.0	162	63.0 ± 5.5^{ab}	29.2 ± 4.3^b	90.0 ± 3.4^a	1.4 ± 0.05^{ab}
50.0	166	52.0 ± 7.4^{ab}	24.0 ± 3.8^b	95.4 ± 2.0^a	1.4 ± 0.06^{ab}
100.0	178	41.8 ± 8.0^b	26.2 ± 4.8^b	92.6 ± 3.5^a	1.3 ± 0.07^b

MNS, mean number sperm.

Within a column, values with different superscripts are significantly different ($p < 0.05$).

Values are expressed as means \pm SEM of 5 replicates.

The percentage of polyspermic oocytes, male pronuclei and MNS/Oocyte are calculated from the number of penetrated oocytes.

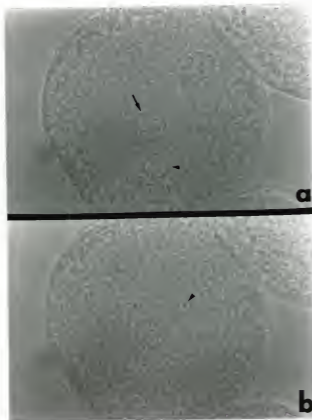


Figure 6-2. Photograph of polyspermic porcine oocyte matured and fertilized without exposure to pOSP.

Panel **A** shows a porcine oocyte with two male pronuclei (arrowhead) and one female pronuclei (arrow). Panel **B** is the same oocyte at a different microscopic field showing the presence of two polar bodies. MPN, male pronuclei, FPN, female pronuclei, PB, polar body. X 40.

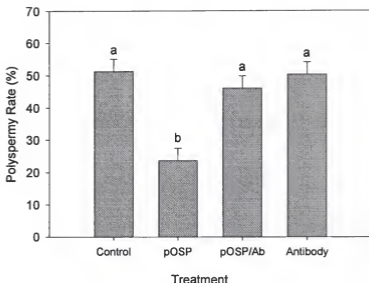


Figure 6-3. Effect of *in vitro* incubation of pOSP and anti-pOSP IgG on the polyspermy rate of porcine oocytes matured and fertilized *in vitro*.

The concentration of pOSP was 10 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$ for the anti-pOSP IgG treatment. A treatment effect ($p < 0.05$) and an interaction ($p < 0.05$) was detected for pOSP and anti-pOSP IgG. Bars indicate the least-squares means \pm SEM of 3 replicates. A total of 210 oocytes are represented for each treatment. Different superscripts indicate significant differences ($p < 0.05$). The rate of polyspermy was determined from the number of penetrated oocytes.

Table 6-2. Effect of pOSP on Zona Pellucida Solubility of Oocytes Matured *In Vitro*.

pOSP ($\mu\text{g/ml}$)	No. oocyte	% Oocytes that Underwent Zona Digestion (minutes)			
		1-3	3-6	6-10	10-20
0.0	35	75.7 \pm 08	15.3 \pm 09	6.7 \pm 6.7	2.3 \pm 2.3
0.1	34	73.3 \pm 10	26.6 \pm 10	0.0 \pm 0.0	0.0 \pm 0.0
10.0	35	80.0 \pm 10	13.3 \pm 04	6.7 \pm 6.6	0.0 \pm 0.0
50.0	34	62.0 \pm 31	31.3 \pm 24	6.7 \pm 6.6	0.0 \pm 0.0
100.0	35	73.0 \pm 16	24.6 \pm 17	2.3 \pm 2.3	0.0 \pm 0.0

Values are expressed as means \pm SEM of 3 replicates.

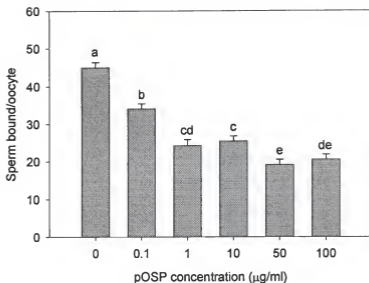


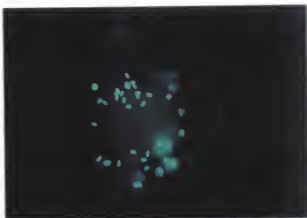
Figure 6-4. Effect of *in vitro* incubation of pOSP on sperm binding to porcine putative zygotes.

Oocytes were *in vitro* matured and then pre-incubated (4 h) and fertilized (6 h) in the presence of increasing concentrations of pOSP (0-100 µg/ml). A treatment effect ($p > 0.05$) was detected for pOSP on sperm binding. Bars indicate the least-squares means \pm SEM of 3 replicates. A total of 45 oocytes are represented for each concentration of pOSP. Different superscripts indicate significant differences ($p < 0.05$).

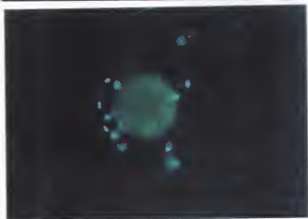
Figure 6-5. Photograph of sperm binding to putative zygotes after pre-incubation and fertilization in the presence of 0 $\mu\text{g/ml}$ pOSP (A) or 100 $\mu\text{g/ml}$ pOSP (B).

Panel B is a representative photograph of the lower end of the range observed for this treatment (about 14 sperm/zygote). Panel A is a representative photograph of the average sperm bound/zygote in the control (about 46). X 200.

A



B



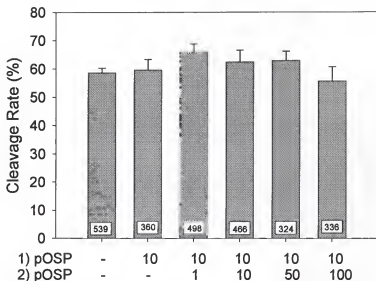


Figure 6-6. Effect of *in vitro* incubation of pOSP on cleavage rate using porcine oocytes matured, fertilized and cultured *in vitro*.

1) *In vitro* matured oocytes were pre-incubated and fertilized in the absence (-) or presence of 10 µg/ml pOSP as described in Materials and Methods. 2) *In vitro* fertilized oocytes were then cultured in the absence or presence of pOSP (1-100 µg/ml) during early cleavage-stage (2- to 4-cell) development. Bars indicate the least-squares means \pm SEM of 9 replicates for each treatment. Shown in each bar are the number of oocytes within each treatment. No treatment effect was detected for pOSP on cleavage rates.

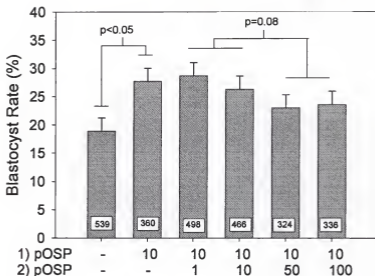


Figure 6-7. Effect of *in vitro* incubation of pOSP on blastocyst development using porcine oocytes matured, fertilized and cultured *in vitro*.

1) *In vitro* matured oocytes were pre-incubated and fertilized in the absence (-) or presence of 10 $\mu\text{g/ml}$ pOSP as described in Methods. 2) *In vitro* fertilized oocytes were then cultured in the absence or presence of pOSP (1-100 $\mu\text{g/ml}$) during embryonic development to the blastocyst. Bars indicate the least-squares means \pm SEM of 9 replicates for each treatment. Shown in each bar is the number of oocytes within each treatment. Differences between treatments were evaluated by orthogonal contrasts and *p* values corresponding to these analyses are shown. Blastocyst formation was calculated as a % of oocytes inseminated (not cleaved embryos).

Discussion

In the present study, it was demonstrated that pOSP administered *in vitro* decreased the incidence of polyspermy in pig oocytes matured and fertilized *in vitro*. This anti-polyspermic effect of pOSP was inhibited by anti-pOSP IgG, indicating that the reduction was specific to pOSP and could not be ascribed to an effector molecule that may have co-purified with pOSP. Polyspermy has remained a persistent problem of pig oocytes matured and fertilized *in vitro*, often reaching levels greater than 50% [Wang et al., 1991, 1994, 1997, Abeydeera and Day, 1997]. However, polyspermic fertilization *in vivo* is uncommon in pigs mated at the onset of estrus prior to ovulation [Hunter, 1967]. A recent study reported that pig oocytes flushed from the oviduct on Day 2 of the estrous cycle and subsequently fertilized *in vitro*, had a much lower incidence of polyspermy (28%) than oocytes matured and fertilized *in vitro* (62%) [Wang et al., 1998]. Likewise, oocytes incubated in the presence of cultured oviductal epithelial cells had a decreased rate of polyspermy [Kano et al., 1994]. In addition, when spermatozoa are co-incubated with oviductal epithelial cells [Nagai and Moore, 1991, Dubuc and Sirard, 1995] or oviductal fluid [Kim et al., 1996] the incidence of polyspermy is reduced significantly. These reports strongly suggest that an unknown factor of oviductal origin can associate with either oocytes and/or spermatozoa, and effectively decrease the incidence of polyspermy. In the pig, the *in vivo* block to polyspermy is thought to be due to a restriction in the number of spermatozoa that reach an egg, and the zona pellucida block to multiple spermatozoa penetration [Hunter, 1990]. However, our findings suggest that a third factor, pOSP synthesized and secreted *de novo* by the oviductal epithelium, may be responsible for the *in vivo* prevention of polyspermic fertilization. Porcine oviduct-

conditioned culture media collected from cultured oviducts of periovulatory gilts was shown to improve *in vitro* maturation, reduce polyspermy, and increase normal fertilization rates, while oviduct-conditioned media from oviducts of luteal gilts did not improve polyspermy or fertilization rates [Vatzias and Hagen, 1999].

Eggs ovulated after luteal phase gonadotrophin treatment show a high incidence of polyspermy (60.6%) and animals treated with progesterone systemically or by local microinjection into the oviduct wall during estrus, show elevated levels of polyspermic fertilization (40% and 32.3%, respectively) [Hunter, 1991]. It has been suggested that under these conditions, the isthmus is more patent allowing more spermatozoa to reach the site of fertilization. During the luteal phase, pOSP mRNA and protein expression are at basal levels, and progesterone has been shown to rapidly down regulate pOSP mRNA and protein synthesis and secretion in ovariectomized gilts [Buhi et al., 1992, 1996]. Thus, the *in vivo* observations noted, may be due to decreased or an absence of pOSP synthesis by the progestational oviduct during fertilization. For most mammals studied to date, *in vitro* fertilization requires large numbers of spermatozoa to efficiently fertilize a high percentage of oocytes. In the case of the pig, these large numbers of spermatozoa lead to a greater incidence of polyspermy [Niwa, 1993]. The observation that *in vitro* administration of pOSP reduces the rate of polyspermy is an important finding for pig IVF, IVF and culture of embryos. However, *in vivo*, where the sperm/egg ratio is very low [Buhi unpublished, Hunter, 1993], the exact nature of pOSP involvement in the block to polyspermy becomes less clear.

In the pig, *in vitro* fertilized oocytes when compared to *in vivo* fertilized oocytes, show an incomplete cortical granule exocytosis [Kim et al., 1996]. It was proposed that a

factor, such as pOSP, might facilitate a more synchronous exocytosis of corticol granule contents or augment the response of the zona matrix to corticol granule exudate. This may be one plausible explanation for pOSPs prevention of *in vivo* polyspermy. However, recent studies utilizing a different maturation system showed no difference in corticol granule exocytosis after fertilization in monospermic or polyspermic eggs [Wang et al., 1998]. Thus, it is unknown why the zona pellucida does not respond *in vitro* as it does *in vivo* to corticol granule exudate. Hamster OSP has been shown to associate with flocculent material in the perivitelline space of oviductal oocytes only after fertilization [Kan and Roux, 1995]. This observation suggested to these investigators that OSP may interact with the contents of corticol granules and facilitate the block to polyspermy. Therefore, corticol granule contents may be interacting with pOSP in the zona pellucida and perivitelline space to modify the ZP receptors and prevent polyspermy.

Oviductal-derived oocytes and embryos have been shown to be more resistant to *in vitro* proteolytic degradation than either follicular oocytes or embryos recovered from the uterine lumen [Broermann et al., 1989]. Similarly, the zona pellucida of *in vitro* matured oocytes are more susceptible to protease digestion by pronase than non-fertilized oocytes flushed from the oviduct on Day 2 of the estrous cycle [Wang et al., 1998]. Therefore, an unknown factor of oviductal origin may be involved in providing zona stability which may also contribute to the functional block to polyspermy. Our results however, suggest that pOSP may not be this factor, as pOSP had no effect on retarding protease digestion of the zona. However, an effect of pOSP on the zona pellucida matrix cannot be ruled out as a titration analysis of various concentrations of pronase was not examined. The concentration of pronase might be too high to detect subtle differences in

modifications of the zona pellucida by pOSP. In addition, this experiment did not include an *in vivo* control (oviductal oocytes) for comparison. Future experiments will include pronase titration analysis and an evaluation of zona hardening after fertilization rather than before fertilization. Modification in the zona pellucida of oviductal oocytes and embryos that provide protease resistance is likely due to known and unknown protease inhibitors of the oviduct, such as PAI-1 and TIMP-1 [Buhi et al., 1996b, Chapters 3-5].

Data in this study indicate that added pOSP decreased the number of spermatozoa bound to the zona pellucida after *in vitro* fertilization. This may be another explanation for the observed decrease in polyspermy. While addition of 0-50 µg/ml of pOSP did not effect penetration of oocytes, it did significantly reduce the number of sperm that attach to the zona pellucida. This function may be the result of a physical block to sperm attachment as pOSP is known to associate with the zona pellucida of ovulated oocytes [Buhi et al., 1993]. Another possibility is that pOSP assists in the modification of zona pellucida sperm receptors immediately after fertilization in a process known as "zona hardening" [Hinsch, 1997]. Corticol granule exudate might therefore interact with pOSP in the conversion of ZP2 and ZP3 to ZP2_f and ZP3_f. These modified zona receptors appear shortly after fertilization and are cleavage products of ZP2 and ZP3, resulting in inhibition of sperm binding [Hinsch, 1997]. Association of pOSP with the zona pellucida [Buhi et al., 1993] may have a role in the initial recognition and binding of spermatozoa by the zona pellucida sperm receptor (ZP1). Porcine OSP, together with ZP1, may assist in a specific selection mechanism of sperm that are highly motile (hyperactivated) and capacitated. Results from these studies appear to be contradictory to findings in the hamster [Schmidt et al., 1997a, 1997b] and human

[O'Day-Bowman et al., 1996]. These studies found an increase in number of spermatozoa bound to the oocyte when exposed to OSP. The differences observed between our data and previous findings may be due to a species-specific difference, purity of OSP, and the use of *in vitro* matured and fertilized oocytes in our study.

The *in vitro* penetration rate of pig oocytes by sperm was not affected by oocyte exposure to pOSP except at a high concentration, where decreased penetration was observed. This high concentration of pOSP and association of large amounts of pOSP with the zona may lead to a physical block of sperm binding. Previously reported data in other species on the penetration rate vary. In the hamster, partially-purified OSP was shown to both increase [Boatman and Magnoni, 1995] and decrease [Kimura et al., 1994] the penetration rate, while in the cow, the penetration rate was found to increase with OSP [Martus et al., 1998]. Differences may be due to means utilized to score penetration rates. In one study, the penetration rate included sperm found within the perivitelline space [Boatman and Magnoni, 1995], while another included eggs having completed meiosis with fused sperm attached [Kimura et al., 1994]. When the penetration rate in the porcine IVF system was low (45-50%), addition of pOSP was observed to increase the penetration rate (65-75%) (data not shown). Bovine OSP (100 µg/ml) was found to increase penetration rates in a cow IVF system (about 51% vs 37% in control) when low concentrations of spermatozoa (0.125×10^6) were used [Martus et al., 1998]. By creating a condition in which the penetration rate was low in the control, an observable increase on penetration rate in the presence of bovine OSP was found. Thus, OSP may be able to maintain high penetration rates after decreasing the number of bound sperm to the zona

pellucida. The increased penetration rate observed *in vitro* by Martus et al. (1998) was due to a specific effect on the oocyte and not the spermatozoon.

This study was not designed to evaluate whether a decrease in polyspermy was due to an effect on one or both gametes. In most domestic species studied thus far, OSP has been shown to associate with the zona pellucida [Boice et al., 1990, Weger and Killian, 1991, Boice et al., 1992, Gandolfi et al., 1991, Murray and Messinger, 1994, Buhi et al., 1993, Leveille et al., 1987]. The association of OSP with spermatozoa is less clear. Species-specific OSPs were found to bind to bovine [King and Killian, 1994] and hamster [Boatman and Magnoni, 1995] spermatozoa, but the human OSP did not associate with human spermatozoa [Reuter, 1994]. There is no evidence to date indicating that pOSP associates with boar spermatozoa. However, incubation of boar spermatozoa with porcine oviductal epithelial cells decreased the incidence of polyspermy in this species [Dubuc and Sirard, 1995]. Further research will be needed to clarify species-specific effects of OSP on individual gametes in relation to fertilization and polyspermy.

Results in this study further demonstrate that pOSP provided a significant increase in post-cleavage development of embryo to blastocyst. This observed increase in blastocyst development may be due to decreased polyspermy. The pathological condition of polyspermy, the penetration of the vitellus by more than one spermatozoa, is known to be a very early cause of death for the zygote [Beatty 1957, Bomsel-Helmreich 1965, Hunter 1991]. However, recent studies indicate that poly-pronuclear pig eggs can develop normally to the blastocyst stage [Han et al., 1999a] and establish pregnancy [Han et al., 1999b]. The addition of pOSP during IVC had no effect on development to

blastocyst, and high concentrations of pOSP tended to decrease the observed effect on development. Physiologically, *in vivo* embryos are no longer exposed to oviductal pOSP after the 4-cell stage that coincides with entry into the uterus in the pig. Therefore, beneficial effects of pOSP on development to blastocyst may have accrued during fertilization and early cleavage-stage cell division (1- to 4-cell). In other domestic species, bovine OSP was found to increase the number of morula and blastocysts on Day 6 but not Day 7 of development [Martus et al., 1998] or had no effect on blastocyst development [Vansteenbrugge et al., 1997]. Similarly, ovine OSP was shown to have no effect on blastocyst development of bovine embryos [Hill et al., 1997]. Thus, a species-specific effect of OSP is observed. The interpretation of many of these studies becomes complicated when preparations of OSP are varied. In this study, and those of Schmidt et al. (1997a, 1997b) and Martus et al. (1998), antibodies specific to that species OSP were used to eliminate the possibility of observed effects being due to co-purified or contaminant proteins. No such experiments have been reported in other studies.

In summary, this study indicates that pOSP significantly reduces the incidence of polyspermy in pig eggs *in vitro* matured and fertilized. This decrease may be due to a reduction in sperm binding to the zona pellucida and not a protective proteolytic-modification of the zona pellucida matrix prior to fertilization. A post-cleavage increase in development to blastocyst was observed when pOSP was included during preincubation/IVF, however addition of pOSP during IVC had no synergistic stimulation on development. These data indicate that pOSP may play an important role *in vivo* in the fertilization process including a block to polyspermy.

CHAPTER 7

SUMMARY AND CONCLUSIONS

The mammalian oviduct has long been recognized for its importance in establishing an optimal environment (pH, temperature, osmotic pressure, nutrients and oxygen tension) for fertilization and early cleavage-stage embryos. Yet, emerging evidence suggests that the oviduct may have a more active role in union of gametes, fertilization, and embryo development. Previous studies have shown that the porcine oviduct invests energy in the *de novo* synthesis and secretion of macromolecules into the oviductal lumen or culture media [Buhi et al., 1997]. The majority of these macromolecules have, until recently, remained unidentified and were characterized in a limited fashion only by molecular weight and isoelectric point. One unidentified protein, of 45,000 molecular weight was shown to be the major secretory product of the isthmus [Buhi et al., 1990].

In Chapter 3, the identification of this unknown isthmic protein was described. A 96% sequence identity was found between this isthmic protein and porcine PAI-1, as determined by N-terminal amino acid sequence analysis. Immunoprecipitation and western blotting further confirmed the identity of this protein. PAI-1, a member of the serpin family of serine protease inhibitors, is the primary inhibitor of the proteases, uPA and tPA. Hence, this protein most likely has an integral role in the regulation of ECM remodeling/degradation and fibrinolysis. Based on previous studies of its activity, the

primary role of PAI-1 in the oviduct should be to regulate PA activation of plasminogen. Both tPA and uPA initiate proteolysis by converting plasminogen to the broad-spectrum enzyme plasmin. Plasmin is responsible for the degradation of fibrinectin and laminin within the ECM and is also capable of activating promatrix metalloproteinases (MMPs). When evaluating PAI-1 function within the oviduct, consideration must be given to the fact that this protein is one inhibitor of a proteolytic cascade leading to the activation of numerous enzymes.

With the identification of PAI-1 in the oviduct, our next objective was to characterize this protein for potential insights into its function. PAI-1 has been shown to be produced by a number of tissues and cells in culture [Andreasen et al., 1990]. Chapter 3 describes the localization of PAI-1 within the pig oviduct. Although this protein is primarily a secretory protein of the isthmus, it can be localized to epithelium of all three segments of the oviduct. Immunocytochemistry revealed that PAI-1 was heavily concentrated in the apical region of epithelium, which suggested possible release into the lumen. No differences could be detected in the localization of PAI-1 between pregnant and non-pregnant animals indicating that its presence in the oviduct was not dependent on the presence of an embryo. PAI-1 was found to be associated with putative secretory granules and cilia of the isthmic epithelium supporting the proposal that this protein is packaged into vesicles for exocytosis and released into the lumen. Localization of PAI-1 in the lumen, near the apical membrane and associated with cilia, may act to: 1) prevent embryonic invasion of oviductal ECM due to oviductal or embryonic PA activity, 2) maintain gamete transport by preventing adhesion of embryos to oviductal fibrin deposits

and 3) regulate the cyclic remodeling of porcine oviductal epithelium in response to the changing steroid environment.

To understand PAI-1's role in relation to fertilization and early cleavage-stage embryo development, expression of PAI-1 mRNA and protein were evaluated during early pregnancy. In Chapter 4, it was shown that synthesis and secretion of PAI-1 protein into culture media varies during early pregnancy (Days 0, 2 and 5). PAI-1 in the Large White was shown to be greatest on Day 2, coinciding with the time of fertilization. The Meishan, numerically showed a greater secretion on Day 0 of early pregnancy, although this value was not significantly different from Day 2. It is unknown why the Meishan would have a greater secretion of PAI-1 on Day 0; possibilities are highlighted in the discussion of Chapter 4. Interestingly, PAI-1 mRNA collected during various days of the estrous cycle in crossbred gilts, were greatest on Day 2 in whole oviduct and isthmus segments. Therefore, expression of PAI-1 mRNA and protein are greatest at the time of fertilization and early embryonic development. Huarte et al. (1993) suggests that there is an important, yet undefined, role for the plasminogen/plasmin system during fertilization. It may be that the elevated expression of PAI-1 at this time contributes to the regulation of this proteolytic cascade. If the proteolytic activity of certain oviductal enzymes, uPA and/or tPA, are not controlled, the zona pellucida matrix could possibly destabilize leading to the possible death of the oocyte or embryo. PAI-1 has a similar mRNA and protein expression to a previously identified metalloproteinase inhibitor of the oviduct, TIMP-1 [Buhi et al., 1996]. The spatial (isthmus) and temporal (Day 2 of early pregnancy) expression of these two inhibitors suggests the importance for some

regulatory measure of proteolysis during fertilization and early cleavage-stage embryonic development.

In the ovary and uterus, PAI-1 has been shown to be regulated by steroids, estrogen and progesterone, and by gonadotropins [Ny et al., 1993, Schatz and Lockwood, 1993]. Chapter 4 describes the regulation of PAI-1 by ovarian steroids in the oviduct. PAI-1 mRNA expression was shown to be stimulated by progesterone while estrogen inhibited this progesterone-mediated effect. PAI-1 protein expression was shown to be inhibited by estrogen, however, the combination of estrogen and progesterone abrogated the effect of estrogen on PAI-1 secretion. In the cycling gilt, estrogen peaks between Days 18-19 and reaches basal levels by Day 1. Therefore, the relief of estrogen inhibition on Day 2 appears to initiate expression of PAI-1 mRNA and protein.

An important question addressed in Chapter 5, "Does oviductal PAI-1 retain biological activity"? It was observed that the majority of PAI-1 was released into culture media in the inactive "latent" form which could be reactivated by denaturants. Denaturant-activated PAI-1 was active functionally and it could: 1) form a complex with uPA 2) be cleaved by uPA in its reactive center and 3) inhibit uPA activity. Additionally, inhibition of uPA activity by PAI-1 was found to be dose-dependent. This evidence suggested that PAI-1 was indeed functionally active once released from the oviductal epithelium. It may be that PAI-1 is stabilized by binding to the proteins in the oviductal or zona ECM.

To evaluate the physiological amount of PAI-1 and PA activity that may be present during early pregnancy, oviduct flushes were obtained on various days (0, 1, 2, 5, 8, 10 and 12). Chapter 5 showed that PA activity was greatest on Day 2 of early

pregnancy. Further examination of this activity using a specific inhibitor of uPA, amiloride, revealed that the majority of this activity is uPA. However, tPA activity may also be present. Use of anti-tPA would be required to confirm this suggestion. Oviductal PAI-1 was also found to be greatest on Day 2 of early pregnancy. In review of these data, it appears that PA activity, PAI-1 mRNA and protein (from explant culture media and oviduct flushes) are elevated during the time of fertilization in the pig.

In light of these results, we suggest that PAI-1 may interact with the oocyte or sperm prior to and during fertilization. To examine this hypothesis, ovulated oviductal oocytes or embryos were collected and evaluated by immunogold electron microscopy. Results of this study are detailed in Chapter 5. PAI-1 was associated with oviductal oocytes and embryos in a similar fashion. The outer rim of the zona pellucida showed intense labeling with PAI-1 while the inner zona, perivitelline space and oocyte or embryo showed moderate levels of PAI-1 localization.

Follicular oocytes did not show the intense labeling of PAI-1 on the outer portion of the zona, indicating that in oviductal oocytes or embryos, PAI-1 was probably obtained from oviductal secretion. Cumulus cells surrounding follicular oocytes were intensely labeled with gold particles, especially in the nuclear region. PAI-1 and PAI-2 mRNA have both been described in bovine cumulus cells [Piquette et al., 1993]. It may be that PAI-1 and/or PAI-2 secretion by cumulus cells protects the preovulatory oocyte from proteolytic degradation due to increased levels of PA at ovulation that are believed to be significant contributors to rupture of the follicle wall through proteolysis of the collagen framework.

Embryos collected from the uterus on Day 4 of early pregnancy (8- to 16-cell stage) show a similar localization of PAI-1 as did oviductal oocytes or embryos (2- to 4-cell). Because the isthmus is the primary source of PAI-1 synthesis and secretion, we hypothesized that the cleavage-stage embryo would sequester PAI-1 from its immediate environment during its transit into the uterus. It cannot be excluded, however, that PAI-1 associated with the uterine embryos is not in part derived from the uterus. One preliminary experiment, not reported here, indicates that PAI-1 may be a primary *de novo* synthesized and secreted product of the Day 4 pregnant porcine endometrium.

Another interesting observation of this study was that PAI-1 associated with the head region of boar spermatozoa when attached to the zona pellucida. Vitronectin, which binds PAI-1, has been shown to be associated with human sperm membranes and is released upon initiation of the acrosome reaction [Fusi et al., 1994]. We propose that oviductal PAI-1 may be binding to vitronectin once released from sperm cells thus stabilizing PAI-1 activity. PAI-1 bound to vitronectin is strategically placed to have an important role in the fertilization process.

The association of PAI-1 with oviductal oocytes and embryos may therefore, prevent: 1) proteolytic degradation of the zona pellucida, 2) premature hatching due to inherent PA activity of the embryo, or 3) adhesion to oviductal epithelium. Additionally, PAI-1 may function in sperm recognition/association, binding and penetration of the zona pellucida.

The second aspect of this proposal was to examine the functionality of a well-characterized porcine oviduct protein, pOSP, during *in vitro* fertilization and culture. In porcine IVF, polyspermy and low blastocyst development (about 30%) remain a major

impediment to the successful culture of pig embryos [Funahashi and Day, 1997]. Several investigators have suggested that oviductal constituents, such as pOSP which are absent in the artificial culture system, may reduce polyspermy and enhance blastocyst development [Hunter, 1991, Dubuc and Sirard, 1995, Kim et al., 1996, Wang et al., 1998]. Chapter 6 describes the results of these experiments. Addition of pOSP to IVM oocytes prior to and during fertilization showed a significant decrease (62% vs 24-29%) in the incidence of polyspermy. Several concentrations of pOSP were found to decrease the rate of polyspermy, and yet did not affect the penetration rate. Higher concentrations of pOSP, however, decreased the penetration rate. This decreased incidence of polyspermy when oocytes are fertilized in the presence of pOSP could be blocked when co-incubated with anti-pOSP IgG. These results indicated that the observed effect on polyspermy was specific to pOSP and not a co-purified product.

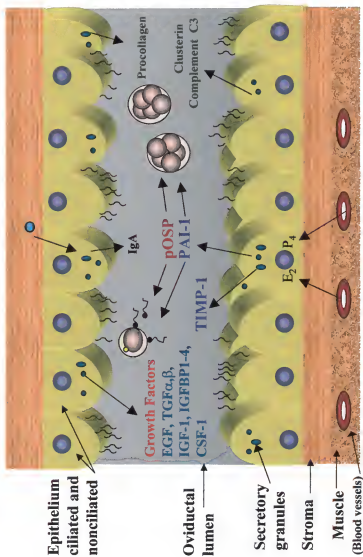
The decreased incidence of polyspermy in oocytes incubated with pOSP was possibly due to an effect on sperm binding. Oocytes exposed to pOSP show decreased numbers of sperm bound to the zona pellucida when compared to controls. Porcine OSP has been shown to associate with the zona pellucida; hence this protein may act as a physical barrier to the binding of multiple spermatozoa. However, there are other possible explanations for this result. One is that pOSP interacts with cortical granule contents after fertilization occurs, leading to a more efficient block of multiple sperm penetrations. This may occur through modifications of the ZP3 and ZP2 receptors causing the release of spermatozoa from their binding sites and inhibition of subsequent sperm attachment. In the hamster, OSP becomes associated with flocculent material in the perivitelline space only after fertilization and is suggested to have a role in the block

to polyspermy [Kan and Roux, 1995]. Another interesting observation of these experiments was that addition of pOSP to IVM oocytes prior to and during fertilization, increased development to blastocyst stage. This was a post-cleavage (2- to 4-cell) phenomenon and may be the result of a decreased incidence of polyspermy. Polyspermy is known to cause the demise of early preimplantation embryos [Beatty, 1957, Bomsel-Helmreich, 1965, Hunter, 1991]. Addition of pOSP, at several concentrations, during IVC had no synergistic stimulation on embryonic development. This suggests that the beneficial effects of pOSP are accrued during fertilization and not cleavage-stage development.

Chapters 3-6 have detailed an active role for the pig oviduct in the synthesis and secretion of proteins that may, through their association with the oocyte or embryo, facilitate the process of fertilization and protect the embryo in a proteolytic rich environment. A developing model of proteins of the pig oviduct is shown in Figure 7-1. Several *de novo* synthesized and secreted proteins of the oviduct have been identified including; PAI-1, TIMP-1, pOSP, clusterin, IgA, procollagen, and Complement C3. Circulating ovarian steroids, such as estrogen, may act to control the expression and release of these proteins in cycle-dependent and stage-specific patterns. Once released into the lumen, these proteins can interact with the oocyte, spermatozoa, or preimplantation embryo and affect their biology. Potential targets include fertilization and cleavage-stage development of oviductal embryos. Further examinations of these proteins will help to elucidate specific roles of the oviduct that may contribute to the previously mentioned processes.

Figure 7-1. Model for the *de novo* synthesis and secretion of proteins by the porcine oviduct.

Several *de novo* synthesized proteins of the porcine oviduct have been identified by N-terminal amino acid sequence analysis or western blotting including; 1) procollagen, 2) clusterin, 3) IgA, 4) complement C3, 5) porcine oviduct-specific secretory glycoprotein (pOSP), 6) tissue inhibitor of matrix metalloproteinase-1 (TIMP-1), and 7) plasminogen activator inhibitor-1 (PAI-1). Also several growth factors and cytokines have been immunolocalized to the epithelium or measured in oviduct flushes by radioimmunoassay. Some of these *de novo* synthesized proteins (PAI-1, TIMP-1 and pOSP) have been shown to be regulated by ovarian steroids, such as estrogen, and have stage-specific, regional-specific, and cycle-dependent expression of their mRNAs. These proteins depicted may interact with the oviductal oocyte, spermatozoa, or early embryo and facilitate the processes of fertilization and early-cleavage stage embryonic development.



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BIOGRAPHICAL SKETCH

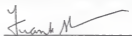
Andrew J. Kouba was born in Omaha, Nebraska, on December 13, 1968. He earned a Bachelor of Science degree at Northwest Missouri State University (NWMSU) with two majors, wildlife ecology/conservation and zoology. After completing his undergraduate degree at NWMSU, Andrew earned a Master's degree in animal physiology at Clemson University, Clemson, South Carolina, in 1995 with a thesis entitled "Insulin-like Growth Factor-1 (IGF-1) in Bovine Seminal Plasma and its Receptor on Spermatozoa," under the guidance of Dr. Don Henricks. He then joined Dr. Bill Bui's laboratory in August 1995 as a Ph.D. student in the Department of Animal Sciences and the Interdisciplinary Concentration in Animal Molecular and Cell Biology at the University of Florida and completed the requirements for the degree of Doctor of Philosophy in December 1999. He will pursue postdoctoral research with Dr. Terri Roth at the Center for Reproduction of Endangered Wildlife (CREW) at the Cincinnati Zoo, Cincinnati, Ohio, beginning October 1999.

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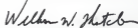
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A handwritten signature in cursive script, appearing to read "Charles W. A.", positioned above a horizontal line.

Dean, College of Agriculture

Dean, Graduate School

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